Effects of Stress on the Barn Owl (*Tyto alba*) and the Link to Melanin-based Coloration

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Summary

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The present thesis investigates the effects of a short period of a chronically elevated corticosterone level, the main stress hormone in birds, in an ecological context and draws a link to the mechanisms explaining individual differences in the sensitivity to stress. The barn owl, as a model species, provides excellent opportunities to investigate parental investment and nestling development in natural conditions and allows experimental manipulations.

I chose an experimental approach to manipulate stress hormone levels in free-living barn owls, using newly available self-degradable pellets releasing corticosterone. These pellets proved to be a powerful tool to elevate corticosterone levels. The hypothalamic-pituitary-adrenal (HPA)-axis responded strongly to the corticosterone administration resulting in a decreased HPA-axis responsiveness as demonstrated by the decreased endogenous response to an acute stressor of corticosterone-implanted birds (chapter 1). Furthermore, the increase of circulating corticosterone after implantation of the pellet and the regulation of free corticosterone through corticosterone-binding globulins (CBG) varied with environmental conditions and food regimes of the nestlings (chapter 6). These results imply that for an understanding of the biological relevance of effects of stress it is not sufficient to study laboratory animals or animals in captivity. On the contrary it is crucial to study free-living animals and observe the context the animal is living in; otherwise we most likely miss or misinterpret important aspects of effects of stress.

Elevated corticosterone levels in breeding barn owl males resulted in a decreased male investment into the brood, which the female did not compensate for, but were not inhibitory to current reproduction (chapter 2). In nestling barn owls a short period of increased corticosterone levels caused a decrease in body mass gain, wing length, and tarsus growth. These reduced growth rates persisted much longer than the corticosterone levels were elevated; at fledging body mass and wing length of corticosterone-implanted nestlings were still lower than in untreated nest mates (chapter 3). Elevated corticosterone levels clearly entailed costs in terms of reduced resistance against oxidative stress, and, because antibody production was reduced, in terms of a higher risk of infections and progression of diseases (chapter 7). All these results together suggest that a short period (only two to three days!) of elevated corticosterone levels influence strongly parental investment and nestling growth and have far longer-lasting effects than the effects that are manifest during the time of elevated corticosterone levels. Such a short period of stress can ultimately shape the phenotype and most likely also influence fitness at adulthood.

I used the hypothesis suggested by Ducrest et al. that eumelanin-based coloration is genetically associated with resistance to stress in vertebrates and formulated the prediction that

darker, more eumelanic, individuals are more resistant to stress. This hypothesis is based on a literature review of genetic and pharmacological studies. Thus it is crucial to test experimentally whether the degree of eumelanin-based coloration is indeed associated with resistance to stress. In this thesis I performed such experiments in breeding males and nestlings. I found that elevated corticosterone levels affected darker individuals less than whiter ones. More eumelanic males reduced provisioning rates less than whiter males (chapter 2), nestling growth was less affected in darker nestlings (chapter 3), and nestlings of darker genetic mothers had lower total and free corticosterone levels after corticosterone administration than nestlings of whiter mothers (chapter 4). These results together support the hypothesis that the degree of eumelanin-based coloration signals the ability to cope with stressful environmental situations and may explain why melanin-based coloration is a mate choice criterion.

We could also demonstrate that elevated corticosterone levels influenced feather phaeomelanin production, which suggests that corticosterone indeed influences the condition-dependent part of melanin production and could therefore reflect the fitness of the individual during feather growth (chapter 5).

The present thesis adds new information on the signaling function of eumelanin-based coloration in the barn owl, as dark owls were significantly better able to cope with an experimental elevation in corticosterone levels than lightly colored owls. Stress sensitivity is an important trait since it influences many other fitness components and when signaled in melanin-based coloration can play a role in sexual selection.

Zusammenfassung

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Die vorgelegte Dissertation untersucht die Auswirkungen von einer leicht erhöhten Corticosteronkonzentration, dem Hauptstresshormon bei Vögeln, in einem ökologischen Zusammenhang und spannt einen Bogen zu den Mechanismen, die den individuellen Unterschieden in der Stresssensibilität zugrunde liegen. Die Schleiereule als Modelorganismus bietet hervorragende Möglichkeiten, den Betreuungsaufwand der Eltern und die Entwicklung der Nestlinge zu untersuchen.

Um eine Stresssituation zu simulieren, habe ich experimentell die Stresshormonkonzentration in frei lebenden Schleiereulen durch Corticosteron ausscheidende Implantate erhöht. Die Implantate erhöhten die Corticosteronkonzentration im Plasma während 2 bis 3 Tagen leicht und bauten sich nach einer gewissen Zeit vollständig ab. Die Hypothalamus-Hypophysen-Nebennierenachse reagierte auf die Corticosteronimplantate mit einer reduzierten Empfindlichkeit. Dies konnte man an der herabgesetzten Corticosteronausschüttung der mit Corticosteron implantierten Vögel als Reaktion auf einen neuen Stressor sehen (Kapitel 1). Es zeigte sich auch, dass der Anstieg von zirkulierendem Corticosteron und die Regulation von freiem Corticosteron durch Corticosteron bindende Proteine (CBG) von den Umweltbedingungen und dem Fütterungsregime der Nestlinge beeinflusst werden (Kapitel 6). Diese Resultate relativieren bisherige Forschungsergebnisse von Labortieren oder Tieren in Gefangenschaft und zeigen die Bedeutung von Untersuchungen an frei lebenden Tieren in ihrer natürlichen Umgebung.

Eine erhöhte Stresshormonkonzentration bei brütenden Schleiereulenmännchen führte zu reduziertem Füttern der Brut, welches nicht durch das Weibchen kompensiert wurde. Im Gegensatz zu gängigen Theorien bewirkt eine erhöhte Stresshormonkonzentration nicht den Brutabbruch (Kapitel 2). Bei Schleiereulennestlingen führte eine kurze Periode von leicht erhöhter Stresshormonkonzentration zu Gewichtsabnahme und reduziertem Flügel- und Tarsuswachstum. Die reduzierte Wachstumsrate war auch noch messbar, als die Stresshormone bereits wieder ihre Ausgangskonzentration erreicht hatten. Zudem waren kurz vor dem Ausfliegen das Körpergewicht und die Flügellänge von Corticosteron implantierten Nestlingen im Vergleich zu den Placebo implantierten Nestlingen immer noch verringert (Kapitel 3). Eine erhöhte Corticosteronkonzentration verursacht klare Kosten für die Nestlinge. Corticosteron implantierte Nestlinge waren weniger resistent gegen oxidativen Stress und hatten ein erhöhtes Infektionsrisiko, verursacht durch die reduzierte Antikörperproduktion (Kapitel 7). All diese Resultate zusammen zeigen, dass eine erhöhte Corticosteronkonzentration während weniger Tage grosse Auswirkungen auf die elterliche Fürsorge und die Entwicklung der Nestlinge haben und den Organismus auch dann noch

beeinflussen, wenn die Stresshormone nicht mehr messbar erhöht sind. Eine solch kurze Stressperiode kann unwiderruflich den Phänotyp und sehr wahrscheinlich auch die Fitness im Erwachsenenalter beeinflussen.

Ducrest et al. (2008) formulierten die Hypothese, dass es einen genetischen Link gibt zwischen Melaninproduktion und Stressresistenz, was dazu führt, dass dunkle, mehr eumelanin gefärbte Individuen stressresistenter sind als hellere Individuen. Diese Hypothese basiert auf einer Literaturstudie von genetischen und pharmakologischen Studien. Darum ist es wichtig, diese Hypothese experimentell zu testen und die Vorhersagen zu überprüfen. In dieser Dissertation führte ich solche Experimente an brütenden Schleiereulenmännchen und Nestlingen durch. Ich fand heraus, dass eine erhöhte Corticosteronkonzentration dunkle Individuen mehr beeinflussten als hellere Individuen. Die Fütterungsrate von dunklen Schleiereulenmännchen war durch eine erhöhte Corticosteronkonzentration weniger reduziert als jene von helleren Männchen (Kapitel 2). Das Wachstum von dunkleren Nestlingen war weniger durch Corticosteron reduziert (Kapitel 3) und Nestlinge von dunkleren Müttern hatten einen weniger starken Corticosteronanstieg nach der Corticosteronimplantation als Nestlinge von helleren Müttern (Kapitel 4). Diese Resultate zusammen unterstützen die Hypothese, dass die Fähigkeit mit einer Stresssituation umzugehen, durch die Färbung gezeigt wird. Auch helfen diese Resultate zu verstehen, warum Färbung, welche auf Melanin basiert, ein Partnerwahl-Kriterium ist.

Zusätzlich konnten wir zeigen, dass eine erhöhte Corticosteronkonzentration die Einlagerung von Phaeomelanin (rotbraune Farbe) beeinflusst. Es scheint tatsächlich so, dass Corticosteron den durch äussere Bedingungen beeinflussbaren Anteil der Melaninproduktion beeinflusst. Die Federfärbung stellt somit die Fitness der Individuen während des Federwachstums dar (Kapitel 5).

Die vorgelegte Dissertation gibt neue Informationen über die Signalfunktion der auf Melanin basierenden Färbung bei Schleiereulen. Dunklere Eulen sind durch eine erhöhte Corticosteronkonzentration weniger beeinflusst als hellere Eulen. Stresssensibilität ist ein wichtiges Merkmal, da viele andere fitnessrelevante Merkmale davon beeinflusst werden. Wenn Stresssensibilität mit der auf Melanin basierenden Färbung signalisiert wird, kann dieses Merkmal durch die sexuelle Selektion ausgewählt werden.

General introduction

General introduction

Genetic and phenotypic variation can be maintained in a population when individuals are differentially adapted to spatially or temporally heterogeneous environments and, on a long-term basis, achieve the same fitness (e.g. Kassen, 2002). Therefore, under large variation in environmental conditions, we may expect not only phenotypic adaptations to certain environmental conditions, but also adaptive genetic variation. To understand the maintenance of adaptive genetic variation it is crucial to investigate the fitness-related traits of different genotypes under alternative environmental conditions. One way is to investigate how different genotypes react to stressful environmental conditions. Differential effects of environmental conditions on different genotypes have been demonstrated for morphological traits (e.g. Merilä and Fry, 1998; Sgro and Hoffmann, 2004; Roulin et al., 2008), but little attention has been paid to the endocrinological basis of such differences. This is important since stressful events including poor nutritional conditions and parasite outbreaks lead to a glucocorticoid response (Kitaysky et al., 2001), which helps the individual to make adequate behavioural responses to overcome the stress situation (reviewed in Sapolsky et al., 2000). However, elevated glucocorticoids can also strongly influence growth patterns, which ultimately shape the phenotype at adulthood (reviewed in Metcalfe and Monaghan, 2001).

In the present thesis I looked at the effects of stress and how these effects varied in different environmental conditions. Further, I investigated whether the differential effects of stress can be explained by genetic polymorphism and whether the stress sensitivity of an individual is signalled in a phenotypic trait. A main aim was to make a link between endocrinology and ecology, an emerging field of interdisciplinary research.

The hypothalamic-pituitary-adrenal axis

Heterogeneity in environmental conditions includes unpredictable weather changes or food scarcity, which are stressful events for animals and require behavioural and physiological adaptations. Such adaptations are induced by a physiological stress response, and the release of glucocorticoids is an important component of this stress response (reviewed in Sapolsky et al., 2000; Charmandari et al., 2005). Glucocorticoid release is part of the hypothalamic-pituitary-adrenal (HPA)-axis. The first wave of the stress response occurs within seconds and includes the secretion of catecholamines from the sympathetic nervous system and the secretion of corticotrophin-releasing hormones (CRH) in the hypothalamus. CRH stimulate the secretion of adrenocorticotropin hormone (ACTH) from the anterior lobe of the pituitary also within seconds. ACTH then triggers the release of glucocorticoids from the adrenal cortex within minutes (Johnson et al., 1992; Sapolsky et al., 2000).

Glucocorticoids play a major role in the regulation of the basal activity of the HPA-axis, as well as in the termination of the stress response through a negative feedback mechanism on the secretion of ACTH and CRH (Charmandari et al., 2005). In many species circulating glucocorticoids consist of a free fraction as well as a fraction that is bound to binding globulins (Westphal, 1983). In birds, the main glucocorticoid is corticosterone, which is bound to corticosterone-binding globulins (CBG). CBG is a glycoprotein specific primarily for glucocorticoids and progestins (it also binds androgens with relatively high affinity) (Westphal, 1983). The free hormone hypothesis (Mendel, 1989) posits that plasma corticosterone bound by CBG is unavailable to enter tissues. Therefore, free unbound hormone would represent the biologically relevant fraction of hormone in the plasma (Westphal, 1983; Mendel, 1989; Rosner, 1990). However, corticosterone bound to CBG may have its own function: at sites of inflammation serine proteases are secreted, cleave CBG which releases corticosterone and thereby increase the local concentration of free corticosterone while maintaining low levels of free corticosterone elsewhere (Pemberton et al., 1988). CBG also binds to membrane receptors and activates intracellular-second-messenger systems (Nakhla et al., 1988; Strel'chyonok and Avvakumov, 1991). However, opinions are mixed and there is still very little evidence directly testing whether in different vertebrate classes total, bound, or free hormone concentration is the relevant fraction. For these reasons it is not sufficient to quantify only total corticosterone; indeed it is necessary to distinguish a free fraction, as well as a fraction that is bound to corticosterone-binding globulins (CBG).

Individual variation in the stress response

Baseline glucocorticoid levels follow a circadian rhythm and are highest around the time of arousal on a daily basis (morning for diurnal species, evening for nocturnal species) (Carsia and Harvey, 2000). In many vertebrates baseline and stress response glucocorticoid levels vary also seasonally with the highest levels during reproduction in reptiles, amphibians, and birds and the lowest in winter (reviewed in Romero, 2002). In mammals seasonal patterns vary greatly between species (Reeder et al., 2006; Nunes et al., 2006; Romero et al., 2008). Furthermore, baseline glucocorticoid levels and levels reached as a response to an acute stress are determined by multiple factors such as sex (Mashburn and Atkinson, 2007), social rank (Muller and Wrangham, 2004), population density (Chapman et al., 1998; Rogovin et al., 2003), and predation risk (Boonstra et al., 1998). Variation in baseline and stress-induced glucocorticoid levels between individuals can be pronounced (Cockrem and Silverin, 2002; Wada et al., 2008) and may correlate with behavioural strategies (Carere et al., 2001; Pfeffer et al., 2002), and reproductive performance (Angelier et al., 2007; Wada et al., 2008).

Researchers have only recently started to investigate whether individual variation in stress response is signalled in phenotypic traits. If stress sensitivity is correlated with a phenotypic trait, sexual selection may favour individuals, which are more stress resistant or have lower stress hormone levels. Mates might choose a partner through condition-dependent traits (e.g. song performance or coloration) or traits that are encoded by genes that pleiotropically alter stress response as suggested by the review of Ducrest et al. (in review). There are several potential reasons why choosing a mate with low corticosterone levels. Short periods of elevated glucocorticoid levels have certainly adaptive functions (e.g. behavioural adaptations to overcome the stress situation, gluconeogenesis to make energy available) (Sapolsky et al., 2000), however chronically elevated stress hormone levels might entail fitness costs. High levels of glucocorticoids suppress certain immune functions (e.g. Harvey et al., 1984; Raberg et al., 1998; Sapolsky et al., 2000), which lead to increased parasite and/or pathogen loads and thus females or males should chose a mate with low glucocorticoid levels in order to chose a healthy mate. High glucocorticoid levels are associated with low body condition (e.g. Wingfield et al., 1994; Jenni-Eiermann et al., 2008) and give therefore a general information of the condition of the animal. Chronically elevated corticosterone levels may lead to reduced parental investment or reduced reproductive behaviour (e.g. Silverin, 1986) or to lower quality of offspring (Sockman and Schwabl, 1999; Love et al., 2004). However, these adverse effects of glucocorticoids have only received little attention in an ecological context (e.g. Kitaysky et al., 2003) and its lifelong fitness costs are mainly unknown.

Examples for the preference for mates with lower corticosterone levels come mainly from two studies on call and song performance. Females of the Great Plains toad (*Bufo cognatus*) preferred males with longer call durations and males, which performed longer calls, had lower corticosterone levels than males with shorter call duration (Leary et al., 2006) Zebra finch (*Taeniopygia guttata*) females preferred males with long song durations and a high complexity of the song performance, and elevated corticosterone levels during development reduced song duration and complexity (Spencer et al., 2003).

Another category of phenotypic traits on which sexual selection may be exerted is melanin-based coloration, since variation in coloration is frequent in vertebrates (Andersson, 1994; Majerus, 1998) and the colour diversity of melanin-based coloration is quite broad, including red (e.g. barn swallow, *Hirunda rustica*), orange (e.g. red jungle fowl, *Gallus gallus*), yellow (e.g. Western tanager, *Piranga ludoviciana*), and green (e.g. mallard, *Anas platyrhynchos*), in addition to black and brown (Jawor and Breitwisch, 2003). Melanin-based coloration is usually heritable and weakly sensitive to environmental condition (Roulin and Dijkstra, 2003; Majerus and Mundy, 2003; Roulin et al., 2004). Furthermore, variation in the degree of melanin-based coloration is correlated with

morphological, physiological, reproductive, and behavioural patterns (Roulin et al., 2004). An association between the response to stress and heritable phenotypic traits has been suggested in Alpine swifts (*Apus melba*) and barn owls (*Tyto alba*) (Roulin et al., 2008). This study did not directly investigate the effect of corticosterone, but applied a stressor (enlarged broods, reduced food supply), but suggests that the ability to cope with stress can be heritable and advertised by melanin-based coloration and thus be potentially subject to sexual selection. There are several possibilities why animals differ in the ability to cope with stressful situations. One possibility is that as a response to a stressor the increase in glucocorticoid levels and/or its duration differs between individuals and therefore the behavioural, physiological, and developmental adaptations differ as well. Another possibility is, that the increase and duration of the glucocorticoid stress response is the same, but the behavioural and developmental adaptations are different depending on the phenotype of the animal. To disentangle these mechanisms experimental manipulations of glucocorticoid levels followed by repeated measures of the hormone levels, behavioural and developmental changes in an ecological context are necessary.

The HPA-axis and melanogenesis

That melanin-based coloration can signal an individual's stress sensitivity is suggested by a direct functional link between melanogenesis and the HPA-axis (Ducrest et al., in review). Melanin consists principally of two heteropolymeres, the brown to black eumelanin and the yellow to reddish-brown phaeomelanin, which are synthesized in melanocytes. Colour differences in melanin-based colorations are, in part, a function of the ratio of the two types of melanin (Jawor and Breitwisch, 2003). In melanocytes binding of melanocortins, in particular α-melanin stimulating hormones (α-MSH), on melanocortin receptors (Mc1-R) triggers eumelanin synthesis. α-MSH is a post-translational product of the pro-opiomelanocortin (*POMC*) gene, which also codes for other melanocortins (adrenocorticotropic hormone (ACTH), β-MSH, γ-MSH, β-lipotrophin (LPH) and γ-LPH). ACTH, as mentioned above, regulates the stress response by binding to Mc2-R, but binds also to Mc1-R together with β –MSH, β- and γ-LPH and induces melanogenesis. Melanocortins bind also to four other melanocortin receptors and regulate e.g. energy homeostasis, immune functions, cardiovascular functions, and sexual behaviour (reviewed in Ducrest et al., in review). Since the same peptides regulate the activity of Mc1-R and the four other Mc-Rs, variation in the levels of these peptides influence the degree of melanism and also other phenotypic traits. For example, in wild vertebrates, blacker and more phaeomelanic individuals are predicted to be less sensitive to stressful factors (reviewed in Ducrest et al., in review). This hypothesis allows deriving predictions about the effects of elevated glucocorticoid levels in more or less melanic individuals.

However, these predictions are based on genetic and pharmacological studies and need to be verified in an ecological context. The present thesis makes such predictions and verifies them with an experimental approach in free-living birds.

Research goals

A main issue in evolutionary and behavioural ecology is to investigate the mechanisms determining the fitness of an individual and its interaction with the environment. Individual variation has often a genetic background and animals are adapted to different conditions. To understand the mechanisms between individual differences and environmental interactions an experimental approach is necessary. In this thesis I performed such an experiment by manipulating corticosterone levels to simulate stressful rearing conditions. Thus, in this study stress refers to physiological stress, which is the reaction of the HPA-axis to a stressor and is measured as the increase of circulating corticosterone levels. The general aim of this study is to investigate the individual differences in the sensitivity to stress in two particular sensitive life-cycle stages, reproduction and postnatal development, under natural environmental conditions in free-living barn owls (*Tyto alba*). The main hypothesis of this thesis is: chronically elevated corticosterone levels have fitness-relevant effects for an individual and these effects can be different between phenotypes. We predict that darker individuals are more stress-resistant than whiter individuals.

Model species barn owl

We chose the barn owl (*Tyto alba*) as study species for several reasons. The barn owl shows naturally large fluctuations in population density, which depends mainly on environmental conditions (Altwegg et al., 2006) and breeds only when conditions are good (Roulin, personal communication). Further it shows large phenotypic variation in phaeomelanin-based coloration from reddish-brown to white and in eumelanin-based coloration from immaculate to heavily marked with black spots of varying size. The expression of these traits is heritable (Roulin and Dijkstra, 2003) and correlated with morphological and reproductive parameters (Roulin et al., 2001; Roulin et al., 2003; Altwegg et al., 2007), and is used as a male mate choice criterion (Roulin, 1999; Roulin, 2004; Roulin and Altwegg, 2007).

Outline of the thesis

In **chapter 1,** we investigated the effects of the corticosterone implants on circulating total and free corticosterone levels and on CBG capacity. This methodological study was necessary because we used as implants biodegradable pellets, which have only rarely been used in birds. The main aim was to determine the duration and levels of the increase of corticosterone in the plasma due to the

corticosterone implants and its effect on the stress response. In this paper we also included data from European kestrel (*Falco tinnunculus*) nestlings.

In **chapter 2,** I investigated the effect of experimentally elevated corticosterone levels in breeding males in relation to melanin-based coloration. In barn owls, males are the sex, which provide most prey items to the offspring and corticosterone is known to reduce parental investment. Based on the hypothesis that the degree of eumelanism signals stress resistance, I predict that elevated corticosterone levels reduce male provisioning rate more in less eumelanic males than in more eumelanic males.

In **chapter 3**, I investigated the effects of corticosterone on nestling growth and whether the effects are linked to eumelanin-based coloration. Corticosterone is known to reduce growth, but little is known about the reasons for individual variation in the reduction of growth due to elevated corticosterone levels. I experimentally elevated circulating corticosterone levels in two of the four experimental nestlings of each brood; the other two received a placebo implant. I predicted that elevated corticosterone levels reduce nestling growth more in less eumelanic nestlings than in more eumelanic nestlings.

Chapter 4: There are two main hypotheses to explain the results found in chapter 2 and 3 about the relationship between melanin-based coloration and resistance to stress. A. Eumelanic individuals are better able to cope with elevated corticosterone levels or B. eumelanic individuals are better able to regulate circulating glucocorticoids, e.g. through a better negative-feedback mechanism or clearance rate. In this chapter I looked whether circulating total and free corticosterone levels differed between more or less eumelanic nestlings before and after corticosterone implantation. I further investigated whether stress-resistance can be inherited through the parents.

In **chapter 5**, we investigated whether corticosterone influenced melanogenesis during feather growth. Based on the negative feedback-link between corticosterone and melanogenesis, we hypothesised that corticosterone mediates the condition-dependent component of melanin production.

In **chapter 6**, I tested the hypothesis that the regulation of chronic stress depends on environmental conditions as well as on body condition. Environmental conditions in 2004 and 2006 were markedly different, with higher food abundance, more breeding pairs, and better nestling body condition in 2004 than 2006. In both years, I performed an experiment with nestlings, where I implanted half of the nestlings with a corticosterone implant and half with a placebo implant as control. Additionally nestlings of 6 broods were fed *ad libitum* in 2004. I was interested to see

whether baseline total and free corticosterone levels and CBG capacity varied with environmental conditions and whether the effects of the implants on total and free corticosterone levels and CBG capacity were different between the years and between different food regimes.

There is a trade-off between the energetic demands of a stress response and other energy demanding functions of the animal such as immune functions. This trade-off may explain why stress-induced corticosterone secretion reduces the strength of immune reactions. In this diploma thesis (**chapter 7**), we investigated the effect of moderately elevated corticosterone levels on the innate constitutive and humoral acquired immune systems in barn owl nestlings.

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Chapter 1

Effects of corticosterone pellets on baseline and stress-induced corticosterone and corticosterone-binding-globulin

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Abstract

Exogenous administration of glucocorticoids is a widely used and efficient tool to investigate the effects of elevated concentrations of these hormones in field studies. Because the effects of corticosterone are dose and duration-dependent, the exact course of plasma corticosterone levels after exogenous administration needs to be known. We tested the performance of self-degradable corticosterone pellets (implanted under the skin) in elevating plasma corticosterone levels by monitoring baseline (sampled within 3 minutes after capture) total corticosterone levels and investigated potential interactions with glucocorticoid-binding-globulin (CBG) capacity and the endogenous corticosterone response to handling in Eurasian kestrel *Falco tinnunculus* and barn owl *Tyto alba* nestlings. Corticosterone pellets designed for a 7-day-release elevated circulating baseline total corticosterone during two to three days compared to placebo-nestlings. Highest levels occurred 1 day after implantation and levels decreased strongly thereafter. CBG capacity was also increased, resulting in a smaller, but still highly significant increase in baseline free corticosterone levels. The release of endogenous corticosterone as a response to handling was strong in placebo-nestlings, but absent 2 and 8 days after corticosterone pellet implantation. This indicates a potential shutdown of the hypothalamo-pituitary-adrenal axis after the two to three days of elevated baseline corticosterone levels. 20 days after pellet implantation, the endogenous corticosterone response to handling of nestlings implanted with corticosterone pellets attained similar levels as in placebo-nestlings. Self-degradable pellets proved to be an efficient tool to artificially elevate circulating baseline corticosterone especially in field studies, requiring only one intervention. The resulting peak-like elevation of circulating corticosterone, the concomitant elevation of CBG capacity, and the absence of an endogenous corticosterone response to an acute stressor have to be taken into account.

Introduction

Exogenous administration of glucocorticoids is a widely used and efficient tool to investigate the effects of elevated concentrations of these hormones. Methods to artificially elevate glucocorticoids include single or repeated injections (e.g. Loiseau et al., 2008), admixture of the hormone to the food or drinking water (e.g. Breuner et al., 1998; Hiebert et al., 2000; Hull et al., 2007) or the insertion of a silastic tube filled with crystalline hormone (e.g. Silverin, 1986; Wingfield and Silverin, 1986; Kitaysky et al., 2001), an injectable gelling material containing hormone (French et al., 2007), a mini-infusion pump (Donker and Beuving, 1989) or an osmotic pump (Horton et al., 2007) under the skin. These various forms have different advantages and disadvantages. Injections imply repeated handling with concurrent internal adrenocortical responses when hormone levels should be elevated over days. While the intake of glucocorticoids with food or water is not invasive, the amount of assimilated glucocorticoids depends on the quantity of food or water ingested and can vary between individuals. Repeated injections and hormone intake with food or water are very difficult to apply in free-living animals. Glucocorticoid-releasing implants or osmotic pumps require only one intervention followed by the release of a given amount of glucocorticoids over a longer period of time and are often the method of choice in field studies. In birds, traditionally silastic tubes filled with crystalline corticosterone have been used. A newer method is the implantation of osmotic pumps. Both of these implants have to be removed after the experiment. An alternative method is the implantation of self-degradable corticosterone releasing pellets, which should provide a constant release by biodegradation of the matrix and do not require subsequent removal. This method has been rarely used in experiments with wild bird species until now (e.g. Pravosudov, 2003; Bourgeon and Raclot, 2006).

Circulating glucocorticoid levels are regulated by a negative feedback mechanism (e.g. Dallman and Yates, 1969; Romero, 2004). Administration of exogenous corticosterone increases circulating corticosterone which then interacts with neural receptors, reducing corticosterone secretion. Further downstream from corticosterone, corticosteroid-binding-globulins (CBG) regulate circulating corticosterone by influencing the general availability of corticosterone to tissues and directing the delivery of hormones to specific sites (e.g. Breuner and Orchinik, 2002). Administration of glucocorticoids has been shown to increase or decrease CBG capacity (Feldman et al., 1979; Zhao et al., 1997).

In most studies the effect of corticosterone administration on circulating corticosterone is not well documented, although the levels of circulating corticosterone attained and the duration of the elevation are supposed to be decisive, since the effects of corticosterone are dose-dependent

(e.g. Romero, 2004) and the duration of the exposure to elevated levels is crucial. The time course of circulating baseline (sampled within 3 minutes after capture) corticosterone after administering corticosterone has been followed in only a few studies by measuring circulating total corticosterone at a few specific time points, e.g. after several days (e.g. Kitaysky et al., 2001). The interactions further up- and downstream from corticosterone during and after an exogenous corticosterone administration have been investigated only rarely (e.g. Feldman et al., 1979; Zhao et al., 1997). We know of no study in birds that investigated the effect of corticosterone administration on CBG or on the response to an acute stressor after the period when circulating corticosterone was elevated

The aim of this paper is to report our experiences with self-degradable corticosterone-releasing pellets in an extended field study with nestling Eurasian kestrels *Falco tinnunculus* and barn owls *Tyto alba*. Specifically, we investigated (a) the time course of total circulating corticosterone during 20 days after implantation; (b) whether CBG capacity and estimated free corticosterone levels were affected by corticosterone administration, and (c) whether the response of circulating corticosterone levels to an acute stressor (handling) was affected by corticosterone administration. We predicted that the negative feedback regulation would decrease the endogenous response to an acute stressor in cort-nestlings with elevated corticosterone compared to placebo-nestlings. For aim (b) and (c) we were interested in the acute effects of the implant and in potential mid-term effects after circulating baseline corticosterone had returned to normal levels.

Material and Methods

Study species and study sites

The Eurasian Kestrel is a small, diurnal raptor. The female starts incubation after laying the third of 4 - 6 eggs, hence, the three oldest nestlings have about the same age. The nestlings stay in the nest for 32 to 39 days and reach their maximal body mass around day 23. Fieldwork was performed in North-western Switzerland (47°25'N, 7°50'E), where kestrels raise their young in nest boxes mounted on agricultural buildings in open rural landscapes. Mean brood size (± SD) at the day of implantation in the investigated broods was 4.4 ± 0.96 nestlings, mean hatching date was on 7th June ± 13 days.

The barn owl is a medium-sized, nocturnal owl species producing clutches of 2 - 11 eggs. Incubation starts after laying the first egg and only females incubate. The laying intervals of two to three days entail a pronounced within-brood age hierarchy. The nestlings reach their maximum body mass with 40 days and fledge with about 56 days of age. Barn owls were investigated in Western Switzerland (46°49'N, 06°56'E), where they breed in nest boxes attached to barns and

farm buildings. Mean brood size (± SD) at the day of implantation in the investigated broods was 5.8 ± 1.54 nestlings, mean hatching date was on 3th June ± 33 days.

Experimental corticosterone treatment

The experiment was carried out in 109 kestrel nestlings of 30 broods (13 in 2004 and 17 in 2005) and 208 barn owl nestlings out of 73 broods (33 in 2004, 19 in 2005, 21 in 2006). Hatching date was determined through regular nest box controls.

Two randomly selected nestlings out of the four oldest within a brood were implanted with a self-degradable corticosterone-releasing pellet on nestling day 13 (mean age ± SD: 13.2 ± 1.4 days) in the kestrel and nestling day 25 in barn owl nestlings. 7-day-release-pellets were obtained from *Innovative Research of America*. Because kestrels were lighter in body mass (mean ± SD: 165 ± 22 g at implantation) than barn owls (294 ± 69 g at implantation), we implanted a 10 mg corticosterone pellet in nestling kestrels and a 15 mg corticosterone pellet in nestling barn owls. The other two of the four oldest siblings were implanted with a corresponding placebo pellet, creating two treatment groups (cort- and placebo-nestlings). Before implantation, body mass (kestrels: $t = 1.44$, $df = 107$, $p = 0.153$; barn owls: $t = 1.56$, $df = 264$, $p = 0.120$) did not differ between cort- and placebo-nestlings. Nestling age at implantation (kestrels: $t = 0.56$, $df = 107$, $p = 0.577$; barn owls: $t = 0.95$, $df = 285$, $p = 0.344$) and sex ratio (kestrels: $\chi^2 = 0.01$, $df = 1$, $p = 0.922$; barn owls: $\chi^2 = 0.09$, $df = 1$, $p = 0.752$) were the same between cort- and placebo-nestling. The pellets were placed under the skin of the flank above the knee through a small incision. The pellets are very sensitive to alcoholic solvents, even when seemingly evaporated. Therefore, to prevent accelerated corticosterone release, the skin was not disinfected. The incision was closed with tissue adhesive (Histoacryl®, Braun, Germany). If more than four siblings were present, the fifth and following were not implanted. All methods described in this study were approved by the Swiss committee for animal research (animal experiment permit n°274 from the Cantonal Veterinarian Office of Baselland for kestrels and n° 1736 from the Veterinarian Office of Vaud for barn owls).

Blood sampling

We took baseline blood samples (within 3 minutes after taking nestlings out of the nest box) in kestrel nestlings three days before implantation, on the day of implantation (day 0) and three and eight days after implantation. In a subgroup of kestrel nestlings we took an additional baseline sample one or two days after implantation. In barn owls we collected a blood sample within 3 minutes after having taken nestlings on the day of implantation and 6 and 20 days after

implantation; a subgroup was also sampled two or three days after implantation. After taking the first blood sample, the nestlings were measured, weighted and then held in a cloth bag. The adrenocortical response to handling was assessed by taking a second blood sample about 17 minutes (16.9 ± 1.4 minutes, range: 14.5 - 22) after taking the nestlings out of the nest box in kestrels and 27 minutes (26.94 ± 7.2 minutes, range: 15 - 48) in barn owls. Blood was sampled by puncturing the alar vein and collected in heparinized capillary tubes. Within 30 minutes, the blood was centrifuged in Eppendorf tubes and the plasma immediately stored in liquid nitrogen in the field and at –20°C once in the laboratory. Corticosterone assay were performed in the next autumn.

Corticosterone assay

Plasma corticosterone concentration was determined using an enzyme immuno assay (Munro and Stabenfeldt, 1984; Munro and Lasley, 1988). 5 µl plasma was added to 195 µl water, and from this solution we extracted corticosterone with 4 ml dichlormethane, which was re-dissolved in phosphate buffer and measured in triplicate in the enzyme-immunoassay. The dilution of the corticosterone antibody (Chemicon; cross-reactivity: 11-dehydrocorticosterone 0.35%, Progesterone 0.004%, 18-OH-DOC 0.01%, Cortisol 0.12%, 18-OH-B 0.02% and Aldosterone 0.06%) was 1:8'000. HRP (1:400'000) linked to corticosterone served as enzyme label and ABTS as substrate. The concentration of corticosterone in plasma samples was calculated by using a standard curve run in duplicate on each plate. Plasma pools from chickens with two different corticosterone concentrations were included as internal controls on each plate. If the concentration was below the detection threshold, the determination was repeated with 10 µl plasma. If the concentration was still below the detection threshold, the value of the lowest detectable concentration (1 ng ml^{-1}) was assigned. Intra-assay variation ranged from 4.5 to 13.4% and inter-assay variation from 9.6 to 23.0%, depending on the concentration of the internal control and the year of determination.

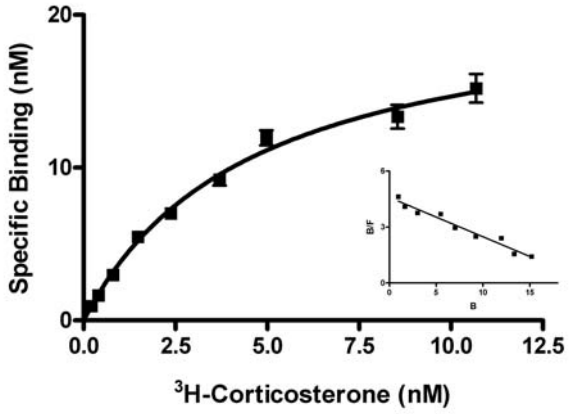
Corticosteroid-binding-globulin

The affinity and capacity of corticosteroid-binding-globulin (CBG) was measured with a radioligand-binding assay with tritiated corticosterone (Breuner et al., 2003). In equilibrium saturation binding experiments on corticosterone-binding-globulin we found a single binding site for corticosterone in kestrel (Kd = 4.59, Fig. 1A) and barn owl plasma (Kd = 4.11, Fig. 1B). For point sample analysis, plasma (10-15 µl in the kestrel, 5 µl in the barn owl) was stripped of endogenous steroids with 2 parts of dextran-coated charcoal (0.1% dextran, 1% Norit A charcoal in 50 mM Tris) for 30 minutes at room temperature. Outside this stripping procedure, the plasma was maintained below 4°C. The final assay dilution of kestrel plasma samples was 1:99, those of the

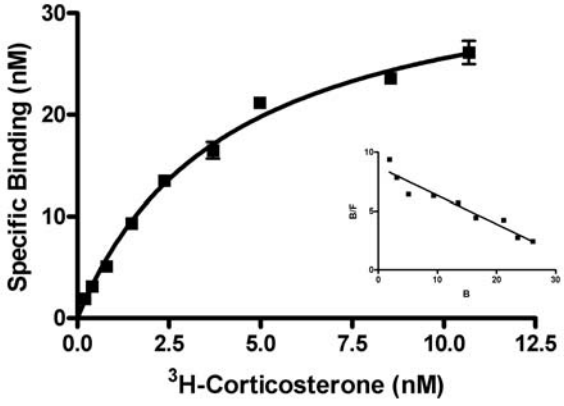
barn owl samples 1:450. The binding assay was carried out in 50 nM Tris buffer at 4°C and terminated after 2 hours. 1 hour before filtering, glass fiber filters (Whatman) were soaked in 25 nM Tris with 0.3% polyethylenimine. After filtration, filters were rapidly rinsed with 3 rinses of 3 ml ice-cold 25 nM Tris. Point sample analysis was performed with individual plasma samples, for the saturation analyses pooled samples were run. For the saturation analyses, 0.25-12 nM [^{3}H]Corticosterone were incubated with pooled plasma of each species with and without additional unlabeled corticosterone. 20 nM [^{3}H]Corticosterone was employed to estimate CBG capacity in individual birds. Affinity estimates, obtained from equilibrium saturation analysis, indicated, that this ligand concentration should occupy ~80% of total binding sites. To account for interassay variation, the samples were corrected to 100% capacity within each assay. The intra-assay variation was 5.7 and 7.1%, the inter-assay variation 7.58 and 17.1% (kestrel and barn owl samples determined separately).

Fig. 1. Equilibrium saturation binding curve demonstrating specific binding of ^{3}H-corticosterone to (A) Eurasian kestrel and (B) barn owl plasma as a function of increasing concentrations of radiolabeled corticosterone. Points indicate means ±SE. The inlay is the Scatchard-Rosenthal replot of the data.

A.



B.



The equation of Barsano and Baumann (1989) was used to estimate free corticosterone titers from total corticosterone concentrations and CBG binding parameters

$$H_{free} = 0.5 \times [H_{total} - B_{\max} - 1/K_a] \pm \sqrt{(B_{\max} - H_{total} + 1/K_a)^2 + 4(H_{total}/K_a)}$$

where H_{free} is free Hormone, H_{total} is total Hormone, B_{max} is total binding capacity of CBG, and K_a = 1/dissociation constant (K_d) (all values in nM). Corticosterone was analysed in all baseline and handling-induced blood samples, CBG capacity was measured in all baseline kestrel and barn owl samples and a subsample of the handling induced kestrel samples (a subsample of day 10, all samples of day 21).

Statistical analyses

To analyse the overall effect of corticosterone treatment on circulating corticosterone we performed mixed-models (REML, Genstat 10) separately with total corticosterone, CBG capacity and free corticosterone as dependent variables. We included treatment (corticosterone *versus* placebo) and time after implantation (number of days after implantation) and their interaction as fixed factors and nestling identity nested in broods as random factors.

The effect of corticosterone treatment on the response of total and free corticosterone and CBG capacity to handling was analysed by running a repeated measure analysis including baseline and stress-induced levels of those days with two blood samples per individual. We included into the fixed model time after implantation, treatment, blood sample (baseline *versus* handling-induced) and their interactions as fixed factors and nestling identity nested in brood as random factors. To compare corticosterone levels of baseline and handling-induced blood samples and CBG capacity between treatment groups at the different sampling days, we performed post-hoc tests for each day separately. Treatment was included as fixed and brood as random factor in these mixed models and significance levels were adjusted according to Bonferroni (Sokal and Rohlf, 2000). Kestrel and barn owl data were analysed in separate models.

Results

Effect of corticosterone pellets on baseline total and free corticosterone levels and CBG capacity

Implanting a corticosterone pellet had a highly significant effect on the plasma concentration of total corticosterone in both species (Table 1, 2). Before implantation, there was no difference in total corticosterone level between cort- and placebo-nestlings in both species (Fig. 2). Total corticosterone in kestrel cort-nestlings was significantly elevated over placebo-nestlings 1 day after

implantation (post-hoc test: $p < 0.001$, Fig. 2, range: 29.78 - 63.80 ng/ml), just not significantly elevated two days after implantation ($p = 0.037$, not significant at the Bonferroni-corrected p-level of 0.013) and indistinguishable from placebo-nestlings 3 and 8 days after implantation. In barn owl nestlings, total corticosterone in cort-nestlings was significantly increased over two days after implantation ($p < 0.001$, Fig. 2, range two days after implantation: 8.11 - 118.03 ng/ml) and returned to the level of placebo-nestlings 3, 6 and 20 days after implantation ($p > 0.161$).

CBG capacity in kestrel nestlings did not significantly vary with time or treatment (Table 1), but there was a non-significant tendency for a higher CBG capacity on day 1 and 2 after cort-implantation in cort-nestlings (Fig. 2; $p = 0.335$). In barn owl nestlings, there was an overall effect of treatment and time and their interaction on CBG capacity (Tab. 2, Fig. 2). CBG capacity of barn owl cort-nestlings was significantly elevated two days after implantation ($p < 0.001$) and indistinguishable from placebo-nestlings 3, 6 and 20 days after implantation.

Estimated free corticosterone levels varied with time and treatment group depending on time after implantation in both species (Table 1 and 2). There was no difference between the treatment groups before implantation in both species (Fig. 2). One day after implantation, free corticosterone was elevated in kestrel cort-nestlings ($p = 0.038$, Fig. 2, not significant at the Bonferroni corrected p-level of 0.013), while 2, 3 and 8 days after implantation there was no difference between the treatment groups. In barn owl nestlings, free corticosterone varied with time after implantation and treatment and their interaction (Table 2). two days after implantation, free corticosterone of cort-nestlings was significantly elevated ($p < 0.001$), while 3, 6 and 20 days after implantation there was no difference between the treatment groups.

Tab. 1. Effect of corticosterone pellets on plasma levels of total baseline corticosterone, CBG capacity and estimated free corticosterone in kestrel nestlings. *Treatment group* refers to 56 nestlings implanted with a corticosterone pellet and 53 nestlings implanted with a placebo pellet. *Time after implantation* refers to blood samples taken 3 and 0 days before and 3 and 8 days after implantation from all nestlings and 1 or two days after implantation from 25 nestlings (470 measurements in total). The results from a mixed model analysis are given with nestling identity nested in broods as random factors.

	Total corticosterone			CBG capacity			Free corticosterone		
	df	F statistic	F pr	df	F statistic	F pr	df	F statistic	F pr
Time after implantation	5, 438.8	29.83	<0.001	5, 308.2	1.44	0.210	5, 320.6	14.37	<0.001
Treatment group	1, 433.5	7.34	0.007	1, 73.7	0.02	0.896	1, 78.2	1.26	0.265
Time after implantation x Treatment group	5, 429.7	33.04	<0.001	5, 314.5	2.05	0.071	5, 323.5	15.78	<0.001

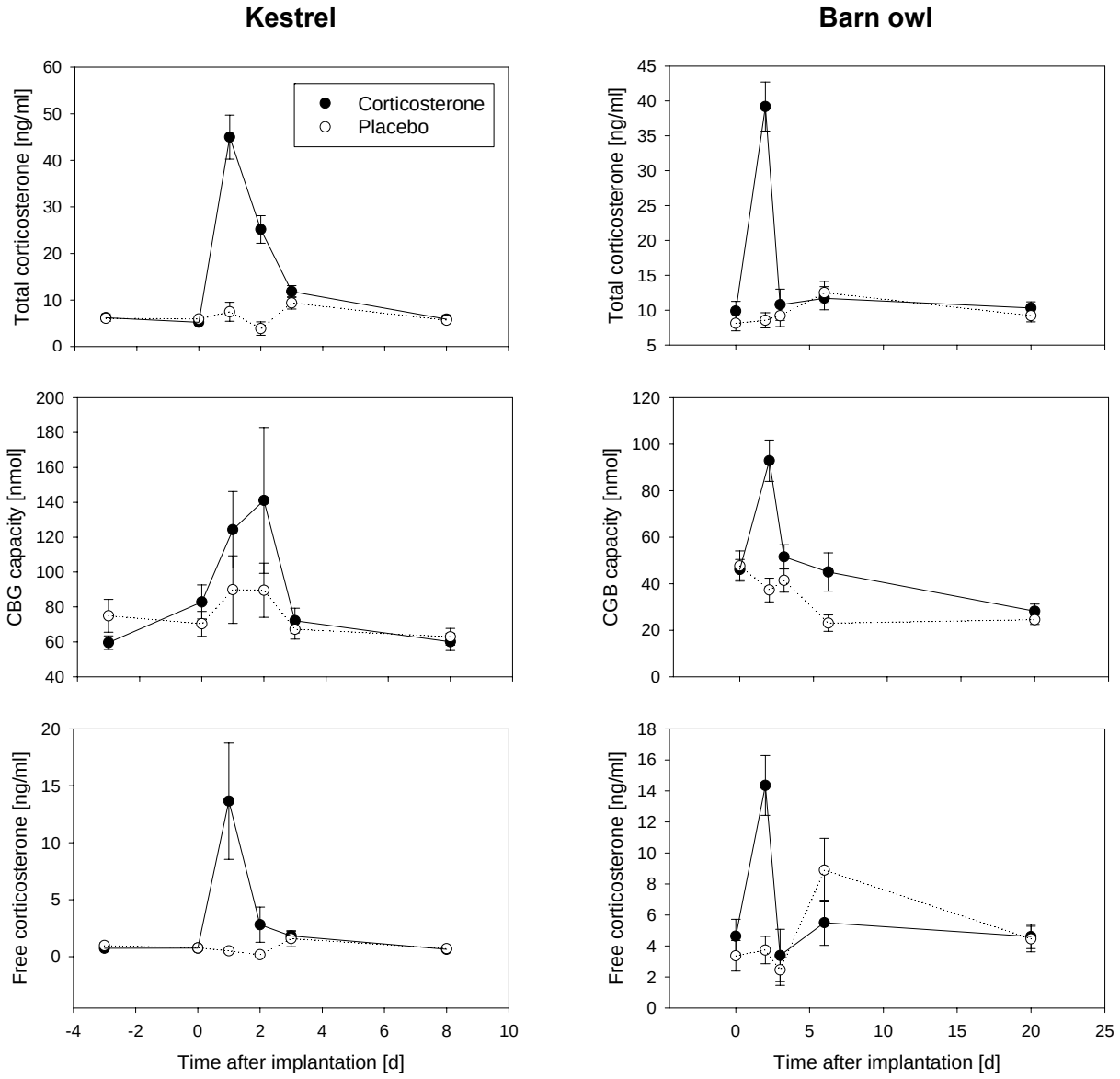


Fig. 2. Effect of corticosterone-releasing pellets on baseline plasma concentration of total corticosterone, on CBG capacity and on estimated plasma concentration of free corticosterone of kestrel (left column) and barn owl nestlings (right column). Indicated are means ± SE of a sample size of 36 to 55 kestrel and 21 to 79 barn owl nestlings per time point and treatment group (cort- and placebo-nestlings) in both species, except on day 1 and 2 after implantation in the kestrel with only 6 samples per treatment group and on day 3 after implantation in the barn owl with only 14 samples per treatment group. The pellet was implanted on day 0, which corresponded to an age of 13 days in kestrels and 27 days in barn owls.

Tab. 2. Effect of corticosterone pellets on plasma levels of total baseline corticosterone, CBG capacity and estimated free corticosterone in barn owl nestlings. *Treatment group* refers to 96 nestlings implanted with a corticosterone pellet and 91 nestlings implanted with a placebo pellet. *Time after implantation* refers to blood samples taken at day 0 (n = 144), 2 (n = 92), 3 (n =28), 6 (n = 47) or 20 days (n = 78) after implantation (389 measurements in total). The results from a mixed model analysis are given with nestling identity nested in broods as random factors.

	Total corticosterone			CBG capacity			Free corticosterone		
	df	F statistic	F pr	df	F statistic	F pr	df	F statistic	F pr
Time after implantation	4, 362.3	28.50	<0.001	4, 270.1	10.52	<0.001	4, 355.2	7.99	<0.001
Treatment group	1, 367.0	46.47	<0.001	1, 113.7	18.59	<0.001	1, 339.4	9.19	0.003
Time after implantation x Treatment group	4, 345.8	28.39	<0.001	4, 288.8	10.15	<0.001	4, 325.5	8.10	<0.001

Effect of corticosterone pellets on handling-induced total and free corticosterone levels and CBG capacity

In both species, total and free corticosterone levels depended on time after implantation, treatment group and blood sample (baseline *versus* handling-induced) (interaction time x treatment group x blood sample $p < 0.001$, Table 3, 4).

Placebo-nestlings of both species showed a marked increase of total plasma corticosterone levels as a response to handling. In cort-nestlings two days after implantation (barn owl, Fig. 3), when baseline total corticosterone levels were elevated, plasma corticosterone levels increased only little as a response to handling and reached similar levels as in placebo-nestlings ($p = 0.130$). In cort-nestlings 8 days after implantation (kestrel, Fig. 3), when baseline levels were low again, the adrenocortical response to handling was virtually absent (interaction time after implantation x treatment group x blood sample $p < 0.001$, Table 3, Fig. 3). In barn owl nestlings 20 days after implantation, when baseline levels were similar in both treatment groups, cort-nestlings reached slightly lower handling-induced levels compared with the placebo-nestlings ($p = 0.053$, Fig. 3).

Tab. 3. Effect of corticosterone pellets on total baseline and handling-induced corticosterone levels in kestrel and barn owl nestlings. *Treatment group* refers to nestlings implanted with a corticosterone or a placebo pellet. *Time after implantation* in the kestrel refers to blood samples taken three days before implantation (n = 106) and 8 days after implantation (n = 103) and, in the barn owl, to blood samples taken at day 0 (n = 75), and 2 (n = 158) or 20 days (n = 51) after implantation. *Blood sample* refers to the baseline sample (taken within 3 minutes) and the handling-induced sample (taken on average 17 (kestrel) or 26 minutes (barn owl) after capture). The results from a mixed model analysis are given with nestling identity nested in broods as random factors.

	Kestrel			Barn owl		
	df	F statistic	F pr	df	F statistic	F pr
Time after implantation	1, 321.7	7.50	0.007	2, 430.2	8.71	<0.001
Blood sample	1, 313.9	155.47	<0.001	1, 395.1	367.96	<0.001
Treatment group	1, 82.6	18.32	<0.001	1, 122.9	3.45	0.066
Time after implantation x Blood sample	1, 313.9	9.67	0.002	2, 396.1	23.05	<0.001
Time after implantation x Treatment group	1, 322.9	39.94	<0.001	2, 476.5	1.15	0.319
Blood sample x Treatment group	1, 313.9	10.75	0.001	1, 395.2	20.90	<0.001
Time after implantation x Blood sample x Treatment group	1, 314.0	29.15	<0.001	2, 396.2	8.25	<0.001

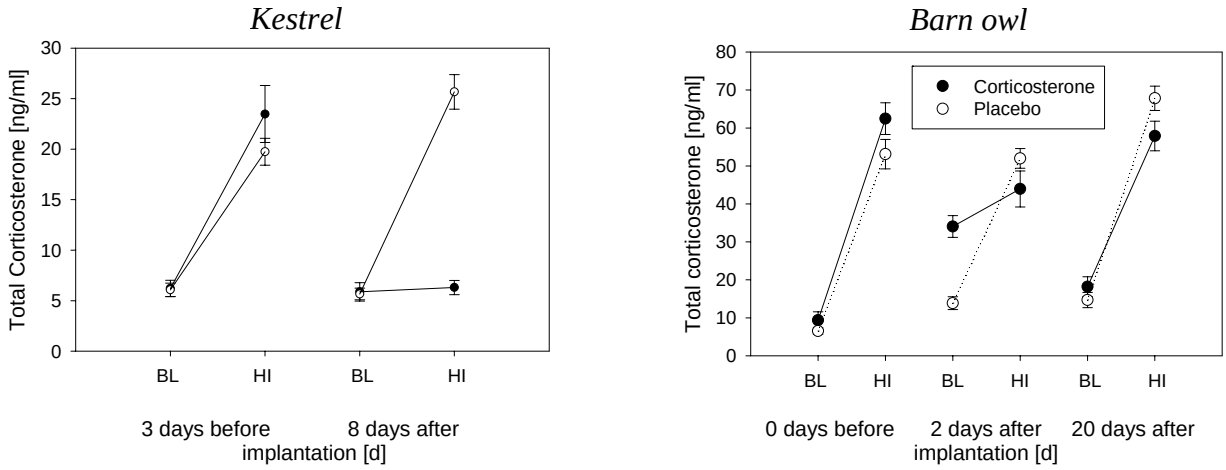


Fig. 3. Baseline (BL) and handling-induced (HI) plasma levels of total corticosterone in kestrel and barn owl nestlings before, 8 (respectively 2 and 20) days after implantation of a corticosterone-releasing pellet. Baseline samples were taken within 3 minutes after capture and handling-induced samples after a mean of 17 minutes in the kestrel and 26 minutes in the barn owl. Indicated are means ± SE of a sample size of n = 45 per time point and treatment group (cort- and placebo-nestlings) in both species. The pellet was implanted on day 0, which corresponded to an age of 13 days in kestrels and about 27 days in barn owls.

Discussion

Effect of corticosterone pellets on total baseline corticosterone levels

The self-degradable corticosterone pellets clearly increased circulating total baseline corticosterone levels in kestrel and barn owl nestlings during two to three days compared with placebo-nestlings. With the 10 mg and 15 mg 7-day release pellets used in this study, the increase in corticosterone levels were similar as those induced after 20 minutes of handling of non experimental birds (kestrel nestlings 10 – 50 ng/ml after 17 minutes; barn owl nestlings 10 - 110 ng/ml after 26 minutes; own unpublished data). However, baseline corticosterone levels were not elevated to a constant level, but peaked one to two days after implantation (no data available 1 day after implantation in barn owls), dropped to a lower level two days after implantation and had almost reached placebo-levels three days after implantation. Thus, corticosterone levels were elevated for a shorter period than the 7 days indicated by the provider. There are two possible explanations. First, the corticosterone contained in the pellet may have been released very fast, resulting in peak-like levels higher than intended. It is possible that the pellet matrix is metabolized faster and corticosterone released in a shorter time period in birds than in mammals, for which the pellet was designed originally. Interestingly, the pellet was still well visible under the skin up to several days after implantation, indicating that the biodegradable carrier-binder was not dissolved completely. A second explanation is that through the internal negative feedback the release of endogenous corticosterone was strongly reduced until the pellet was metabolized. The finding that the glucocorticoid response to handling was strongly reduced up to 8 days after implantation may suggest that the negative feedback mechanism was indeed in operation (see below). Future studies, using pellets with labelled corticosterone, are needed to determine the relative amounts of endogenous and exogenous corticosterone in the blood and, thus, the importance of the internal negative feedback mechanism to regulate plasma levels of corticosterone after external administration.

In a study implanting silastic tubes filled with corticosterone in starlings *Sturnus vulgaris* and black-legged kittiwakes *Rissa tridactyla*, plasma corticosterone levels also had decreased to near placebo levels three or five days after implantation (Romero et al., 2005; Angelier et al., 2007), also indicating a potential negative feedback regulation reducing the adrenocortical corticosterone release after some days. Studies using other techniques to administer corticosterone unfortunately do not present the time course of circulating corticosterone after administration. Usually, the resulting circulating corticosterone levels were measured only once at varying time points after the

beginning of administration, which prevents the comparison of the time course of corticosterone levels between different methods.

Effect of corticosterone pellets on CBG capacity and free baseline corticosterone levels

Concurrent to the increased corticosterone levels the pellets also entailed a significantly higher CBG capacity in barn owl nestlings and a tendency to a higher CBG capacity (small sample size) in kestrel nestlings. This corresponds to increased serum CBG in mouse pups after glucocorticoid administration (Zhao et al., 1997), but is in contrast to a decrease in CBG production and secretion in rats after glucocorticoid administration (Feldman et al., 1979). It is possible that the increase in CBG capacity serves to protect partly from the deleterious interferences of high free corticosterone levels with postnatal morphological and cognitive development (Kitaysky et al., 2003). However, the course of free baseline corticosterone correlated strongly with total baseline corticosterone levels, and the peak in total corticosterone concentration after pellet implantation was buffered only to a small degree by the simultaneously elevated CBG capacity. We did not find any impact of the corticosterone pellets on CBG capacity after the peak of circulating baseline corticosterone, indicating no potential direct effect of corticosterone administration on CBG regulation.

Effect of corticosterone pellets on the adrenocortical response to handling

There was only a small adrenocortical response to handling two days after corticosterone pellet implantation in barn owl nestlings and virtually no adrenocortical response to handling 8 days after corticosterone pellet implantation in kestrel nestlings. This can be explained by the negative feedback mechanism controlling circulating corticosterone levels (e.g. Keller-Wood and Dallman, 1984; McEwen et al., 1986). An attenuated adrenocortical response to an acute stressor occurs when the animal is already under chronic stress as provoked here by corticosterone administration. The hypothalamo-pituitary-adrenal axis shuts down and is not capable of mounting a response to an acute stress (Romero, 2004). One example in free-living birds is the absence of any further corticosterone elevation to capture and handling in a seabird, when a severe storm had already substantially increased circulating corticosterone (Smith et al., 1994).

The negative feedback can occur as a rate-sensitive fast feedback and a level-sensitive delayed feedback (Dallman and Yates, 1969). The delayed feedback begins approximately 30 minutes following glucocorticoid elevation and extends for days. The duration of the subsequent inhibition of the axis depends on the absolute concentration of the steroids, thus may extend beyond the period of hormone administration (e.g. Abe and Critchlow, 1980; Sapolsky et al., 1986). Therefore, the absence of an adrenocortical response to handling in kestrel nestlings eight days after

corticosterone pellet implantation (when baseline total corticosterone levels were not elevated anymore) may be explained by such an extension of a level-sensitive delayed feedback. Alternatively, it may be explained by the continuous release of corticosterone from the pellet provoking the shut-down of the hypothalamo-pituitary-adrenal axis, as mentioned above.

Twenty days after corticosterone pellet implantation, the HPA-axis seemed to have recovered from the corticosterone manipulation. As a response to handling barn owl cort-nestlings showed a similar increase in circulating total corticosterone levels as placebo-nestlings.

Conclusions

Self-degrading pellets are an efficient tool to artificially elevate corticosterone levels with one intervention. The dose and duration-dependent effects of corticosterone require the monitoring of the resulting circulating corticosterone levels. It is to be expected that the release from pellets, and possibly from other implants, is not constant over time or that a delayed feedback sets in after a few days which both result in different plasma levels, and thus possibly different effects, of corticosterone at different stages during the experiment. The plasma levels resulting from corticosterone administration could be monitored in a subgroup within the experiment, in an additional group of implanted control animals or in a preliminary study. However, since the HPA-axis can vary with season, life history stage, environmental conditions, age and sex (e.g. Wingfield et al., 1994; Kitaysky et al., 1999; Romero, 2002; O'Reilly and Wingfield, 2003; Love and Williams, 2008), any additional group should closely match the experimental groups and live under similar conditions and at the same time or season.

The interactions of exogenous corticosterone with the negative feedback mechanism and the response to an acute stressor have to be taken into account in future studies. An increase in CBG capacity provoked by corticosterone administration results in an attenuated increase in circulating free baseline corticosterone levels compared with total levels, which may modify the effects of corticosterone on the animal. The level-sensitive delayed negative feedback in glucocorticoids, suppressing an endogenous corticosterone response to an acute stressor, can extend up to several days after artificially elevated plasma corticosterone levels have returned to normal levels.

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Chapter 2

Parental investment and its sensitivity to corticosterone is linked to melanin-based coloration in barn owls

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Abstract

Behavioral and physiological responses to unpredictable changes in environmental conditions are, in part, mediated by glucocorticoids (corticosterone in birds). In polymorphic species, individuals of the same sex and age display different heritable melanin-based color morphs, associated with physiological and reproductive parameters and possibly alternative strategies to cope with variation in environmental conditions. We examined whether the role of corticosterone in resolving the trade-off between self-maintenance and reproductive activities covaries with the size of melanin-based spots displayed on the ventral body side of male barn owls. Administration of corticosterone to simulate physiological stress in males revealed pronounced changes in their food provisioning rates to nestlings compared to control males. Corticosterone-treated males with small eumelanic spots reduced nestling provisioning rates as compared to controls, and also to a greater degree than did corticosterone-treated males with large spots. Large-spotted males generally exhibited lower parental provisioning and appear insensitive to exogenous corticosterone suggesting that the size of the black spots on the breast feathers predicts the ability to cope with stressful situations. The reduced provisioning rate of corticosterone-treated males caused a temporary reduction in nestling growth rates but did not affect fledgling success. This suggests that moderately elevated corticosterone levels are not inhibitory to current reproduction but rather trigger behavioral responses to maximize lifetime reproductive success.

Introduction

The resolution of the trade-off between self-maintenance and reproductive effort is mediated by physiological mechanisms, such as the hypothalamic-pituitary-adrenal (HPA)-axis. Environmental perturbations may activate the HPA-axis and lead to the release of glucocorticoids, which help mobilize stored energy, redirect behavior to self-maintenance and enhanced restfulness at the expense of reproductive investment (reviewed in Sapolsky et al., 2000). While a short-term release of glucocorticoids is considered beneficial in allowing individuals to overcome stressful situations and reestablish homeostasis (Wingfield and Kitaysky, 2002; McEwen and Wingfield, 2003), chronically elevated levels can entail negative long-term effects (Sapolsky et al., 1986; Sapolsky et al., 2000). Evidence for an adaptive role of corticosterone comes from experimental studies where a short-term elevation of corticosterone level increased survival (Meylan and Clobert, 2005; Cote et al., 2006).

The responsiveness of the HPA-axis to environmental perturbation changes seasonally (Schwabl et al., 1980; Romero, 2002) and can vary between individuals (Cockrem and Silverin, 2002). Inter-individual variation in physiological, hormonal and behavioral traits can result not only from variation in condition, but may also be the outcome of selection having favored alternative heritable strategies to cope with unpredictable changes in key environmental factors. Accordingly, individual coping styles can have a genetic component and be associated with consistent differences in endocrine and behavioral responses to stressful situations. For example, aggressive pigs explore a novel object sooner than non-aggressive pigs and show an increase in cortisol levels, whereas in non-aggressive pigs cortisol levels remain low (Hessing et al., 1994). Great tits (*Parus major*) of two different selection lines for coping styles (slow vs. fast explorers) show different levels of corticosterone secretion when confronted with an aggressive resident male (Carere et al., 2003).

Assuming that inter-individual variation in coping styles is heritable, as the above examples suggest, selection may favor the evolution of phenotypic traits that advertise the ability to overcome stressful conditions. This situation may have evolved several times in different color-polymorphic species, whose individuals display alternative heritable morphs that are associated with difference in survival, behavior, physiological, and reproductive parameters (review in Roulin, 2004c). Each color morph can therefore reflect alternative strategies to cope with environmental conditions that fluctuate in space and time. An example for the coexistence of morphs is the tawny owl (*Strix aluco*) in which grey females produce offspring of higher quality while reddish-brown females breed more often (Roulin et al., 2003b). Most examples of color polymorphism come from species for which variation in coloration is due to differential deposition of melanin pigments raising the

possibility that alternative melanin-based color patterns are associated with different physiological adaptations to cope with stressful environmental factors (Roulin et al., 2008). This is plausible because there is a functional link between the glucocorticoid response to environmental stressors and melanogenesis. Production of melanin pigments is, amongst other, regulated through the melanocortin 1-receptor, its agonists the melanocortins melanin-stimulating-hormones (MSH) and adrenocorticotropic hormone (ACTH) which are post-translational products of the pro-opiomelanocortin (*POMC*) gene (Slominski et al., 2004). Melanocortins not only regulate melanogenesis, but also other physiological functions including the stress response mainly through ACTH (Slominski et al., 2006).

In the present study, our aim is to investigate whether the role of corticosterone in resolving the trade-off between self-maintenance and reproductive activities covaries with the degree of melanin-based coloration. We chose the barn owl (*Tyto alba*) as study species because individuals from the same population vary in the degree of eumelanin-based coloration, from a complete absence of black spots to being heavily marked with spots and in the degree of phaeomelanin-based coloration from reddish-brown to white. This offers us the opportunity to examine whether an experimentally elevated level of corticosterone affects parental investment differently in alternatively colored individuals. This is plausible because in the barn owl, melanin-based coloration is associated with several behavioral, morphological, and physiological characteristics that have been linked to the ability to cope with stressful factors including heart size (Roulin et al., 2001a), feeding rate of nestlings (Roulin et al., 2001a), foraging method (Roulin, 2004d), recruitment into the population (Roulin and Altwegg, 2007), parasite resistance (Roulin et al., 2001b) and developmental homeostasis (Roulin et al., 2003a).

We tested whether the effect of experimentally elevated corticosterone levels in breeding males, the sex that provides most prey items to the offspring, was color-dependent. Our method of administering corticosterone allowed us to manipulate the mediator of the stress response (i.e. corticosterone), in the absence of stressful environmental factors. Because corticosterone stimulates foraging for self-maintenance (Silverin, 1986; Wingfield and Silverin, 1986; Lohmus et al., 2006) and reduces parental investment (Silverin, 1986; Silverin and Wingfield, 1998; Love et al., 2004), we predicted that corticosterone administration would reduce males' provisioning rates. Based on the hypothesis that the degree of eumelanism (but not of phaeomelanism) signals the ability to cope with stressful factors (Roulin et al., 2008), we also predicted that males displaying smaller black spots reduce provisioning rate following an experimental increase in circulating corticosterone more than males with larger black spots. Finally, we determined how the manipulation of corticosterone levels in breeding males affected the growth of their offspring.

Material and Methods

Study organism

The barn owl is a medium-sized predator of small mammals (99% of the diet, Roulin, 2004a). The two to eleven eggs are laid between February and August, and eggs hatch asynchronously on average every second or third day creating a pronounced within-brood age hierarchy. Only females incubate the eggs and thus we can recognize them from males by the presence of a brood patch. Nestling growth rate peaks at 17 days post-hatch and nestlings are heaviest at 40 days post-hatch, from which point they gradually lose weight until fledging at ca. 56 days. Three-week old nestlings can thermoregulate and, hence, are no longer brooded by their mother whose daytime roost is at some distance from the nest. Extra-pair paternity is rare (Roulin et al., 2004). Variation in plumage coloration is already visible in nestlings. Although individuals of the two sexes can express any phenotype, males are, on average, less reddish-brown and display less and smaller black spots than females. All birds become slightly lighter in color when molting body plumage between the first and second year of life (Roulin, 1999a), but a bird that was darker than another individual in its first year is still darker in its second year (Roulin and Dijkstra, 2003). Plumage coloration and spottiness are genetically correlated within both sexes (spottier individuals are darker reddish-brown; Roulin, 2004b) and between the sexes (darker fathers and darker mothers produce darker sons and daughters, spottier fathers and spottier mothers produce spottier sons and daughters; Roulin et al., 2001a).

Experimental manipulation of corticosterone level

The study was carried out in an area of 190 km^2 located at an altitude ranging from 430 to 520 m in Western Switzerland (46°49'N, 06°56'E) in 2005. The area is dominated by agriculture and holds a barn owl population of 20-80 pairs breeding in 110 nest boxes put in barns. To experimentally investigate the effect of moderately elevated plasma corticosterone levels on provisioning rate, we captured 41 male barn owls at night when they were feeding their 24 ± 3 days old offspring (mean ± SE). Within 3 minutes of capturing a male, a blood sample was taken to determine the baseline plasma corticosterone level (Romero and Reed, 2005); we were able to blood samples 36 of 41 males within 3 minutes. Blood samples were taken by puncturing the brachial vein and collected into heparinized capillary tubes. Samples were centrifuged within 15 minutes of collection and the plasma immediately stored in liquid nitrogen in the field and at –20°C once in the laboratory in the evening. We determined plasma corticosterone concentrations with an enzyme-immunoassay (Munro and Stabenfeldt, 1984; Munro and Lasley, 1988) after extraction of 5 µl plasma and 195 µl water in 4 ml dichlormethane. All samples were run in triplicates. The dilution of the corticosterone

antibody (Chemicon; cross-reactivity: 11-dehydrocorticosterone 0.35%, progesterone 0.004%, 18-OH-DOC 0.01%, cortisol 0.12%, 18-OH-B 0.02% and aldosterone 0.06%) was 1:8'000. HRP (1:400'000) linked to corticosterone served as enzyme label and ABTS as substrate. The concentration of corticosterone in plasma samples was calculated by using a standard curve run in duplicates on each plate. Plasma pools from chicken with a low and a high corticosterone concentration were included as internal controls on each plate. Intra-assay variation ranges from 5 to 13% and inter-assay variation from 12 to 21%, depending on the concentration of the internal controls.

After blood sampling, we implanted 21 males with a corticosterone pellet (hereafter cort-male) and 20 others with a placebo pellet (placebo-male). The pellets (diameter 5 mm) are made up of a biodegradable carrier-binder containing 15 mg corticosterone or, for placebo, only of the biodegradable carrier-binder (Innovative Research of America, Sarasota, Florida). We implanted the pellet under the skin of the flank above the knee through a small incision, which was closed with tissue adhesive (Histoacryl, Braun, Germany). We could not determine the increase in plasma corticosterone levels in the experimental males, because repeated captures would have caused serious disturbance possibly increasing the risk of brood abandonment. However, the company specified that pellets release corticosterone tonically over a seven-day period in rats. From a previous unpublished study in 2004 with nestling barn owls, which have a similar body mass as breeding males, implantation of the same implants increased circulating corticosterone levels by about 18 ng/ml (plasma baseline corticosterone levels before implantation ± SE: 10 ng/ml ± 1.13, two days post-implantation: 28 ng/ml ± 2.81, six days post-implantation: 12 ng/ml ± 2.16). Following an acute stressor (i.e. handling), barn owls increase plasma corticosterone levels up to 60 ng/ml (B. Almasi, unpublished data) and therefore the effect of the corticosterone implants can be considered as within the physiological range. The Swiss committee for animal research approved the study (animal experiment permit from the 'Service vétérinaire du canton de Vaud' n° 1736).

Assessment of plumage traits

In males, we scored breast phaeomelanin-based coloration by comparison with eight color chips from 1 for dark reddish-brown to 8 for white. From a previous study we know that two measures of coloration taken on a same individual are highly correlated (female: $r_S = 0.95$, $p < 0.001$, $n = 125$ individuals; male: $r_S = 0.96$, $p < 0.001$, $n = 31$), and thus the method of assessing this plumage trait is repeatable (Roulin, 1999b). We also counted black spots located near the tip of feathers within a 60 x 40 mm area on the breast and measured their diameter to the nearest 0.1.mm. A mean spot diameter value was calculated and used for analysis. The repeatability of measuring number of

spots in a recent study was 0.93 in breeding males (one-way ANOVA: $F_{29,36}$=26.54, P<0.0001) and 0.89 in breeding females ($F_{194,174}$=15.96, P<0.0001), and the repeatability of measuring spot diameter 0.92 in breeding males ($F_{29,36}$=24.88, P<0.0001) and 0.84 in breeding females ($F_{91,140}$=11.45, P<0.0001) (Roulin, 2004b). The age of twenty breeding males was known precisely as we banded them as nestlings (n= 20). Unknown aged males were classified as 'yearling' if all primary and secondary feathers belonged to the same generation, and as 'adult' otherwise (Taylor, 1993). In our sample of pairs, male and female partners did not resemble each other with respect to plumage coloration (Pearson correlation: df = 39, r = 0.05, p = 0.76) and spot diameter (df = 39, r = 0.19, p = 0.29). Laying-date was not correlated with male and female plumage traits (p-values > 0.09).

Assessment of parental provisioning rate

The night after having implanted males, we started to record provisioning rates during four successive nights using infrared cameras installed outside or inside the nest boxes. The position of the cameras had no effect on male and female provisioning rates (Student's t-test: p-values > 0.36). Provisioning frequency of males and females, banded on a different leg to recognize them on the video footage, was defined as the number of prey items brought to the nest box from 10 p.m. until sunrise. Since prey species differ markedly in body mass, we estimated total prey mass delivered per night. To this end, we identified prey items on video footage either as voles (416 items), woodmice (156) or undetermined (450). Because it was difficult to identify common voles (*Microtus arvalis*) from water voles (*Arvicola terrestris*) from video footage, for each nest we identified prey remains found during daylight hours (in total we found 11 ± 6 prey remains per nest) and calculated the proportion of voles that were common voles (72.7%) and water voles (27.3%). Based on a previous study on prey remains found in barn owl nests, common voles weigh on average 29.1 g, water voles 49.2 g and woodmice 33.5 g (Roulin, 2004c). To obtain an estimate of the amount of grams of prey brought by males and females each night, we used the following formula: $provisioning\ rate = n \times (v \times vm + m \times mm)$, where n is the number of prey items brought to the brood per night (provisioning frequency), v is the percentage of voles brought to the brood per night determined on video footage, vm is the mean vole mass calculated from the proportion of common voles and water voles of prey remains, m is the percentage of woodmice brought to the brood per night determined on video footage, and mm is the mean woodmouse mass. Due to technical failures and nestlings sitting in front of the cameras we obtained provisioning rates from 102 nights in the 41 experimental nests.

Assessment of nestling growth

To investigate the indirect effect of manipulating corticosterone level in breeding males on their offspring, we weighed all 212 nestlings to the nearest 0.1 g the day before males were captured, as well as all surviving nestlings at 6 and 27 days after implantation. For each nestling we calculated mean body-mass gain per day from day 0 to day 6 and from day 6 to day 27. The rank of each nestling in the within-brood age hierarchy was determined around hatching, a time when we clipped off the tip of one claw to recognize each individual until being ringed with an aluminium ring at ca. two weeks of age. There were no differences between the corticosterone and placebo treatments in mean hatching date, brood size on the first day of the experiment, mean nestling age, male and female plumage traits (Student's *t*-tests: all p-values > 0.13), and male and female age (Pearson's Chi-squared test: p-values > 0.58).

Statistical procedure

To test the hypothesis that individual variation in provisioning rate of cort- and placebo-male barn owls is linked to plumage traits, we performed a repeated mixed effect model analysis. We included into the model male provisioning rate as the dependent variable, male identity as a random factor to control for pseudo-replication, the three categorical variables treatment (corticosterone vs. placebo), night (nights 1, 2, 3 and 4 post-implantation) and male age (yearling vs. adult), and the four covariates date, male body condition (male body mass divided by cubic wing length), phaeomelanin-based coloration and spot diameter. Since spot diameter and number of spots correlated significantly (Pearson correlation: $n = 41$, $r = 0.774$, $p < 0.001$), we considered only spot diameter; we chose spot diameter instead of number of spots because this trait is more often associated with other phenotypic traits than number of spots (Roulin, 2004a). The best models were selected based on Aikaike's Information Criterion for small sample size (AICc) (Akaike, 1974; Burnham and Anderson, 2002), where the model with the lowest AICc-value is the most parsimonious. To avoid having an unreasonably large number of models, we first built models with the variables male age, night, number of nestlings, date and male treatment and their biologically relevant interactions ('night x treatment', 'male age x treatment', 'male body condition x treatment'). Models with a $\Delta AICc < 2$ compared to the model with the lowest AICc were selected to be expanded in the second step by including male plumage traits (color score and spot diameter). All models with a $\Delta AICc < 2$ were chosen for model averaging to estimate the predicted means and their SE. For model averaging we calculated Akaike model weights (ωi) (Anderson et al., 2000) which indicate the probability that a given model is the best among the whole set of candidate models. Weights of models sum up to 1 by definition. The model with the highest weight is

considered as the best. Predicted parameters are multiplied by the weight of the particular model and summed over all selected models to give the weighted average of the predicted parameters (Burnham and Anderson, 2002). We calculated standard errors (SE) of the predicted values with the bootstrap method using 5'000 iterations (Crawley, 2006). We also used model averaging to obtain model-averaged effect sizes for main effects without interactions and their SE.

To test for a potential effect of male treatment (cort- vs. placebo-implanted males) on offspring, we applied analogous repeated mixed effect models with offspring body-mass gain as the dependent variable. We first included male treatment, growth period (0-6 days, 6-27 days after the start of the experiment), and nestling rank in the within-brood age hierarchy as categorical variables, and date and number of nestlings in the brood as covariates. In a second step, we expanded the best models with male and nestling phaeomelanin-based coloration and spot diameter. We included site and nestling identity nested in site as random factors to account for repeated measurements of the same nestling. The averaged predicted parameters were calculated as described above.

Results

Individual variation in baseline corticosterone

Baseline levels of circulating corticosterone before treatment did not differ between cort- (11.12 ng ml^{-1} ± 0.9) and placebo-males (14.4 ng ml^{-1} ± 1; Student's t-test: t = -1.542, df = 33, p = 0.133) and was not correlated with male phaeomelanin-based coloration (Pearson correlation: n = 36, r = 0.136, p = 0.465) or spot diameter (n = 36, r = -0.396, p = 0.695).

Effect of male corticosterone treatment on provisioning rate

Model selection revealed two best models to explain variation in the effect of male corticosterone treatment on male provision rate. The first model included night, male treatment, age, body condition, and spot diameter as well as the interactions 'male spot diameter x male treatment' and 'male spot diameter x male age'. The second best model in addition included the interaction 'male treatment x male body condition' (Table 1 A). Corticosterone treatment reduced male provisioning rate with cort-males feeding on average 68 g of prey mass per night less than placebo-males (mean prey mass per night ± S.E. 281 g ± 16 versus 349 g ± 21). Males with smaller spots brought generally more food, but were more affected by the corticosterone treatment than males with larger spots (interaction male spot diameter by male treatment). Cort-males with small spots reduced their provisioning rate to the level of placebo-males displaying large spots (Fig.1 C & D). Age alone and in interaction with male spot diameter had a small positive effect, which was more pronounced in

larger-spotted males, i.e. young larger-spotted males fed less than older males whereas the provisioning rate of young and old smaller-spotted males was the same. Body condition had a small positive effect on provisioning rate and the interaction of body condition and male treatment was only included in the second best model.

Model selection showed that females did not compensate for the decrease in provisioning rate of cort-males (Table 1 B). The two best models revealed that female provisioning rate increased slightly with date (model-averaged effect size ± SE: 1.982gd^{-1} ± 0.847) and decreased with female age (effect size ± SE: -32g ± 44).

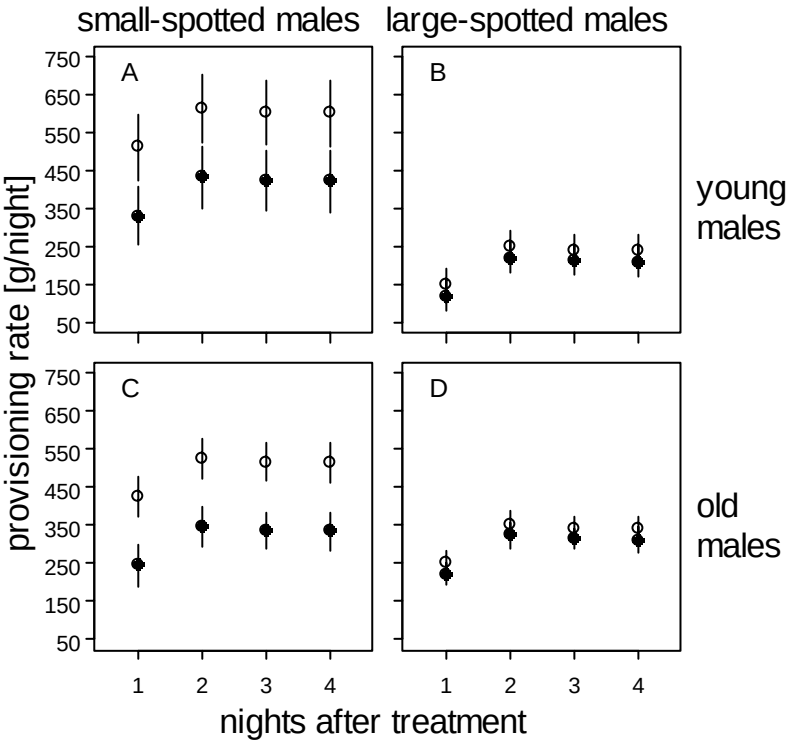

Fig. 1. Model-averaged predicted male provisioning rate in g night^{-1} (± SE) after treatment of (A) first year males with small spots, (B) first year maleswith large spots, (C) males older than one year with small spots and (D) males older than one year with large spots. The continous variable spot diameter ranged from 0 to 2.1 mm. For 'small-spotted males' spot diameter 0.1 mm was entered into the model and spot diameter 1.3 mm for 'large-spotted males'. Open symbols represent placebo-implanted males, closed symbols corticosterone-implanted males, respectively. These figures are based on the number of feedings per night in 41 pairs over 102 nights. There are significant differences in the provisioning rates of untreated small-spotted and large-spotted males. Cort-treated small-spotted males decreased their provisioning rates significantly compared to large-spotted males.

Effect of male corticosterone treatment on nestling growth and survival

Overall, male provisioning rates correlated significantly with body mass gain of the entire brood during the time of the male stress challenge (growth period 0 to 6, Pearson's correlation: $r = 0.501$, $n = 41$, $p = 0.002$). The reduced food-provisioning rate by cort-males during the first six days resulted in a reduced body mass growth rate (2.98 gd^{-1} ± 0.76 per nestling raised by cort-males versus 7.16 gd^{-1} ± 0.70 per nestling raised by placebo-males; Table 2). From day 6 to day 27 growth rate was lower than during the first six days and there was no difference in body mass gain between nestlings raised by cort- and placebo-males.

Post-hoc, we examined whether male plumage traits were associated with the ability of offspring to cope with the food restriction caused by a lower provisioning rate of cort-males. Model selection yielded three best models with similar AICc and Aikaike's weights. All models included male treatment, nestling rank in the within-brood age hierarchy, and growth period as explanatory variables. The two best models also included male phaeomelanin-based coloration and the interaction of male phaeomelanin-based coloration with treatment (Table 2). The reduction in body-mass gain during the first six days post treatment in nestlings raised by cort-males was more pronounced when the father was reddish-brown (Fig. 2 A) than when he was whitish (Fig. 2 B). Nestling phaeomelanin-based coloration and spot diameter as well as father spot diameter were not included in the best models. Note that food-provisioning rate did not differ between males of different color (Table 1 A), although it clearly varied with spot size (see above).

Only four out of 212 nestlings died (1.9%) during the first four days after having implanted a corticosterone-releasing pellet in males precluding any analysis of an immediate effect of male corticosterone treatment on nestling survival. Between the start of the experiment and fledging a total of 30 nestlings died out of 212 (14.2%), but these mortality events were not associated with male treatment (Pearson's Chi-squared test: $\chi^2 = 0.232$, $df = 1$, $p = 0.630$). Thus, cort- and placebo-males produced a similar number of fledglings (4.6 fledglings ± 0.2 of cort-males versus 4.9 fledglings ± 0.3 of placebo-males; Student's *t*-tests: $t = -0.658$, $df = 39$, p-values = 0.514).

Table 1. Model selection of repeated mixed-effect models to explain variation in male and female provisioning rates in relation to male corticosterone treatment (t), male spot diameter (md), male age (a), male body condition (c), female age (fa), number of nestlings (cl), night (n) and date (d). Male identity was introduced as a random factor. Log-Likelihood (LogL), number of estimated parameters (K), Akaike's information criterion (AICc), difference of AICc to the best model (ΔAICc) and Aikaike's weight (ωi) from the 5 best models are reported. This model selection is based on the measurement of number of the feedings per night in 41 pairs over 102 nights. The best models (ΔAICc < 2) are in bold.

	Model	Variables	LogL	K	AICc	Δ AICc	ωi
A. males	**1**	**c+a+n+t+md+md*t+md*a**	**-623.64**	**12**	**1274.78**	**0.00**	**0.34**
	2	**c+a+n+t+md+c*t+md*t+md*a**	**-622.43**	**13**	**1274.99**	**0.21**	**0.31**
	3	c+a+n+t+md+md*a	-625.92	11	1276.78	2.00	0.13
	4	c+a+n+t+md+c*t+md*t	-624.93	12	1277.37	2.59	0.09
	5	c+a+n+t+md+c*t+md*a	-625.12	12	1277.75	2.97	0.08
B. females	**1**	**d**	**-617.71**	**4**	**1243.84**	**0.00**	**0.39**
	2	**fa+d**	**-617.44**	**5**	**1245.50**	**1.66**	**0.17**
	3	fa+d+t	-616.91	6	1246.71	2.87	0.09
	4	cl	-619.38	4	1247.16	3.32	0.07
	5	fa+cl+t	-617.27	6	1247.42	3.58	0.06

Table 2. Model selection of repeated mixed-effect models to explain variation in body-mass gain in relation to father corticosterone treatment (t), father phaeomelanin-based coloration (mc), father spot diameter (md), rank (r), date (d) and growth period (v). Site and nestling identity nested in site were introduced as random factors. Log-Likelihood (LogL), number of estimated parameters (K), Akaike's information criterion (AICc), difference of AICc to the best model (ΔAICc) and Aikaike's weight (ωi) from the 5 best models are reported. This model selection is based on the 392 measures of body mass gain in 212 nestlings. The best models (ΔAICc < 2) are in bold.

	Model	Variables	LogL	K	AICc	Δ AICc	ωi
body-mass gain	**1**	**d+r+t+v+v*t+mc+mc*t+mc*v**	**-1202.24**	**17**	**2440.12**	**0.00**	**0.35**
	2	**r+t+v+v*t+mc+mc*t+mc*v**	**-1203.97**	**16**	**2441.39**	**1.28**	**0.18**
	3	**d+r+t+v+v*t**	**-1206.40**	**14**	**2441.90**	**1.79**	**0.14**
	4	d+r+t+v+v*t+mc+mc*t	-1204.84	16	2443.13	3.02	0.08
	5	d+r+t+v+v*t+md	-1206.00	15	2443.27	3.15	0.07

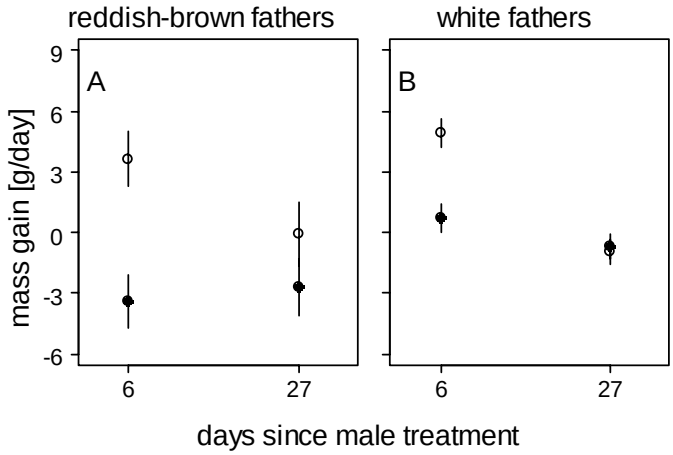

Fig. 2. Model-averaged predicted body-mass gain of nestlings in g day^{-1} (± SE) over the first six days after father treatment and from day 6 to day 27 after father treatment. The continous variable father phaeomelanin-based color which ranged from 1 (dark reddish-brown) to 8 (white) is set in (A) at 3.6 (reddish-brown) and (B) at 7 (white). Open symbols represent nestlings of placebo implanted fathers, closed symbols nestlings of corticosterone implanted fathers, respectively. These figures are based on the 392 measures of body mass gain in 212 nestlings. From day 0 to day 6 of the experiment body mass gain of nestlings of cort-treated reddish-brown fathers was lower than body mass gain of nestlings of cort-treated white fathers, whereas body mass gain of nestlings from different morphs of untreated fathers did not differ. There was no difference between the groups from day 6 to day 27.

Discussion

In this study, we demonstrated that the administration of corticosterone to breeding males decreased their provisioning rates, and that their mates did not compensate for it. The depression in provisioning rates in the first night after treatment in all males may be due to the implantation procedure or more likely due to the attachment of radio-tags, which were used for another study. The effect of exogenous corticosterone on provisioning rates was more pronounced in small-spotted males, with large-spotted males showing not only a minimal response to corticosterone but an overall lower level of provisioning. The reduced provisioning rate of corticosterone-treated males entailed a temporarily reduced growth rate in their nestlings, that was consistent with the estimated life-span of the implants, but no reduction in breeding success. Nestlings may cope differently with a reduced food supply depending on their fathers' phaeomelanin-based coloration.

Effect of corticosterone on provisioning rate

An effect of corticosterone on reproductive effort has been previously reported. As in barn owls, a moderate administration of corticosterone in male and female pied flycatchers (*Ficedula hyploeuca*) reduced feeding frequency and a higher dose provoked the abandonment of the brood (Silverin, 1986). In contrast, in black-legged kittiwakes (*Rissa tridactyla*) corticosterone-treated parents

(either male or female) spent more time away from the brood without decreasing feeding rate (Kitaysky et al., 2001). Thus, corticosterone administration may differentially affect behaviors across species and, therefore, one might expect different species to react differently to environmental perturbations. We suggest that an increase in corticosterone level alters the most plastic behaviors of parental investment. In the kittiwake with only two chicks in need of guarding in an open nest, a modulation of time–budget seems to be used as a buffer against environmental variability (Angelier et al., 2007a), rather than a reduction of the feeding frequency. In contrast, the barn owl and the pied flycatcher have a variable number of nestlings (mostly 5 – 8) in a protected cavity in no need of guarding and parents are continuously in search for food. At least in barn owls, environmental variability is mainly charged to the brood and hardly buffered by the parents, because the parents are already working at their sustainable maximum (e.g. Roulin et al., 1999). Thus, when corticosterone is changing the trade-off between reproductive effort and self-maintenance in favor of the latter, kittiwakes reduce guarding, exposing the chicks to a higher risk of predation, while barn owls and pied flycatchers reduce feeding frequency, thus exposing the chicks to a higher risk of starvation. We do not know whether male barn owls increased foraging for their own need after corticosterone administration, but findings in the pied-flycatcher (less body-mass loss during the feeding period in corticosterone treated birds; Silverin, 1986), the black-legged kittiwake (increased time spent away from the brood; Kitaysky et al., 2001) and the wandering albatross (*Diomedea exulans*) (corticosterone levels correlate positively with daily distance traveled during a foraging trip; Angelier et al., 2007b) suggest an increase in foraging activity of these birds and a higher investment in self-maintenance at the expense of parental investment. This conclusion is consistent with the observation that prolactin levels, a hormone that regulates nest bond and parental behavior (Cherel et al., 1994; Buntin, 1996; Criscuolo et al., 2005; Chastel et al., 2005; Criscuolo et al., 2006), decrease when corticosterone levels increase in birds (Cherel et al., 1994; Criscuolo et al., 2005; Chastel et al., 2005; Criscuolo et al., 2006).

Under natural conditions, food shortage (possibly due to bad weather) and the presence of predators are the most common unpredictable environmental factors leading to a raise in corticosterone. When environmental conditions deteriorate, the costs to rise a brood successfully increases and, consequently, parents should invest more in their own condition and survival and, thus, in future reproduction. When corticosterone levels increase and reach a certain threshold the brood will most probably be abandoned (Silverin, 1986). If corticosterone levels are only moderately elevated (and below this certain threshold), elevated corticosterone levels may not be inhibitory to current reproduction, but rather trigger behavioral responses to maximize lifetime reproductive success of the parents.

Melanin-based plumage coloration and coping with increased corticosterone levels

Our study is among the few that focused on individual variation to a physiological stress response (Carere et al., 2001; Pfeffer et al., 2002; Carere et al., 2003; Wada et al., in press). We showed that there is pronounced difference in parental investment between different phenotypes and further that there are pronounced differences in how birds with different phenotypes cope with physiological stress (in our study simulated by administering corticosterone). The untreated smaller-spotted males invested more into reproduction, but when corticosterone levels increased, they were more stress-sensitive, than the larger-spotted males with a lower parental investment and a lower susceptibility to stress.

Baseline circulating corticosterone levels were not related to male melanin-based traits and the associated parental investment while a short-term increase in corticosterone produced differential behavioral responses in differently colored individuals. We do not know whether baseline corticosterone levels mediate parental investment in different morphs, but a behavioral response might not only be due to circulating levels of corticosterone but also depend on receptor type and receptor density, which can be different between the morphs.

Our study showed that individual differences in coping with stress are signaled with a phenotypic trait, here plumage eumelanin-based coloration. The less stress-sensitive males displayed larger black spots than the more stress-sensitive males. We obtained similar results in barn owl nestlings in that more eumelanic nestlings and nestlings with more eumelanic mothers showed lower plasma corticosterone levels after corticosterone administration (B. Almasi, in preparation), suggesting a stronger negative feedback mechanism or clearance rate of these individuals. The same mechanism might explain the better stress-resistance of darker eumelanic males. In a similar line, nestlings raised by foster parents developed more symmetrical feathers of the left and right wings when their biological mother displayed larger black spots (Roulin et al., 2003a). Finally, under stressful rearing conditions nestling Alpine swifts (*Apus melba*) grew more rapidly only if their biological father was darker eumelanic (Bize et al., 2006). These various studies suggest that the degree of eumelanin-based coloration is associated with the ability to cope with stressful situations.

That less eumelanic birds are more stress-sensitive agrees with the hypothesis of a functional link between melanogenesis and melanocortins, including ACTH (Racca et al., 2005; A.L. Ducrest, L. Keller, A. Roulin, submitted). Such a functional link may be most operational during feather growth (i.e. in chicks and during molt in adults), but may reflect a more general individual variation

in physiology. In the males of this study, molt of remiges regularly started during the period of chick feeding.

The link between stress sensitivity and plumage coloration may contribute to the maintenance of genetic variation in eumelanin-based coloration in this population. In favorable environmental conditions more stress-sensitive, less eumelanic individuals may have a higher reproductive output or chicks of better quality through their higher parental investment, while in sub-optimal environmental conditions less stress-sensitive darker eumelanic individuals may attain a higher fitness, because they may abandon their brood less easily and because they may be genetically predisposed to resist stressful environmental situations. In this study, phaeomelanin-based coloration seems not to be involved in signaling the ability of an individual to cope with stress, a finding that is consistent with a recent study (Roulin et al., 2008).

Effect of increased corticosterone levels in fathers on nestling growth

Administration of corticosterone to free-living male barn owls decreased provisioning rates to their brood by about 70 gram per night (i.e. the mass of 2.5 voles per night), which was not compensated for by the female. Not surprisingly, this food restriction resulted in a reduced body-mass gain in the nestlings compared with control broods.

The provisioning rate of fathers did not vary with the fathers' phaeomelanin-based coloration. However, when the fathers were stressed, fathers displaying more reddish-brown coloration produced offspring, which gained less body mass than offspring of whiter fathers. (Table 2). This is the result of a correlative post-hoc analysis and lacks experimental rigor (e.g. controlling food supply to the nestlings and cross-fostering nestlings to allocate genotypes randomly among rearing environments), but it points to two phenomena that lead to interesting further questions.

First, not eumelanin-based coloration, the trait, which correlated with stress sensitivity of the fathers, but phaeomelanin-based coloration correlates with body mass gain of the nestlings under food restriction. Thus, different plumage characters may signal different aspects of stress sensitivity. Furthermore, the heritability of stress sensitivity may be linked in a complex way with plumage characteristics and possibly other characteristics of the bird. This calls for a comprehensive recording of plumage characters in further studies.

Second, because the provisioning rate of both placebo- and cort-males did not vary with phaeomelanin-based coloration, variation in food supply cannot explain the variation in body-mass gain of chicks with cort-fathers. Different body-mass gain, when food supply is similar, may result from differences in energy-allocation between nestlings of more or less phaeomelanic fathers. For

instance, under food restriction chicks of phaeomelanic fathers may allocate more energy into tissue maturation or immune function while chicks of white fathers try to maintain body-mass gain as best as they can. Reducing metabolic rate may be another means of maximizing growth.

These two observations call for further studies to elucidate (1) how stress coping styles are signaled in various plumage traits and (2) whether fathers pass on genes resulting in different strategies to cope with food restriction in chicks, which, however, are not detected in the chicks' coloration (there is no direct effect of nestling phaeomelanin-based coloration on their body mass gain).

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Chapter 3

Genotypic polymorphism, signaled by melanin-based coloration, affects stress induced phenotypic flexibility

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Abstract

A growing body of evidence indicates that some genotypes are better adapted to stressful environmental conditions than others. A key issue is to understand why one genotype may better resist stress than another. Ducrest et al. (2008) proposed the hypothesis that in vertebrates in which inter-individual variation in coloration is due to variation in melanocortin levels, dark eumelanic individuals are more resistant to stress than lighter colored conspecifics. In the present study, we experimentally tested predictions derived from this hypothesis. To this end, we artificially administrated in nestlings, the mediator of stress responses in birds, corticosterone, to examine the prediction that corticosterone-induced reduction in growth rate is more pronounced in lightly colored nestlings than in darker nest mates. The specific prediction is that the difference in growth rate between corticosterone- and placebo-implanted nestlings covaries with the degree of eumelanin-based coloration. We found that under corticosterone administration, nestlings with large black spots grew better than nestlings with smaller black spots. As the expression of eumelanin-based coloration in the barn owl is heritable and not sensitive to environmental variation, this trait is therefore a reliable genetically-based signal of resistance to stress-induced increase in blood corticosterone level.

Introduction

Most natural environments are heterogeneous in space and time, a critical parameter for the evolutionary stability of many genetic polymorphism (Kawecki and Ebert, 2004; Hedrick, 2006). A growing body of evidence indeed indicates that different genotypes are adapted to alternative environmental conditions (Nachman et al., 2003; e.g. Savolainen et al., 2007) implying that spatial or temporal variation in environmental conditions allows each genotype to achieve the same fitness on the long term (Kassen, 2002; Byers, 2005). For example, conditions prevailing in one habitat or in one year may be more favorable for one genotype, while conditions occurring in another habitat or year may be better suited for another genotype. A key issue is to identify the biotic or abiotic factors to which genotypes are locally adapted. In many instances, environmental heterogeneity is variation in habitat quality which may select for genotypes that are able to cope with stressful situations induced for instance by parasites and food scarcity (e.g. Roulin et al., 2001). Inter-individual variation in stress resistance can be due to heritable variation in the glucocorticoid response to an acute stressor (Carere et al., 2001; Pfeffer et al., 2002) since glucocorticoids play an important role in orchestrating an adaptive response to suboptimal or stressful environmental conditions (Hofer and East, 1998; Charmandari et al., 2005). Understanding why one genotype better resists stress than another requires the identification of a candidate gene that can explain variation in reaction norms between genotypes across habitats that vary in the level of stress. Here we report a case where knowledge of the physiological effects of a candidate gene is useful to generate *a priori* predictions regarding which of two genotypes should be more resistant to stressful environmental factors.

Vertebrates often vary in the degree of melanin-based coloration, a trait that is often under strong genetic control (Majerus, 1998; Gantz and Fong, 2003; Roulin and Dijkstra, 2003 but see Griffith et al., 1999; Horth, 2003; Fargallo et al., 2007). In many species coloration is associated with morphological, physiological, reproductive and behavioral parameters. This association suggests that dark and lightly colored individuals may be adapted to different environmental conditions (Jawor and Breitwisch, 2003; Roulin, 2004a). Of particular interest is the finding that in several species darker eumelanic individuals better resist stressful conditions than less eumelanic conspecifics (Roulin et al., 2008; Almasi et al., 2008). For instance, blacker feral pigeons (*Columba livia*) were more resistant to the effects of the nuclear accident in Chernobyl (Johnston and Janiga, 1995), blacker Harris' sparrows (*Zonotrichia querula*) had lower levels of blood corticosterone (Rohwer and Wingfield, 1981), Alpine swift (*Apus melba*) offspring of darker eumelanic fathers grew their wings more rapidly in experimentally enlarged broods than offspring of less dark fathers (Roulin et al., 2008), darker siskins (*Carduelis spinus*) were less susceptible to stressful conditions

as measured by metabolic rate (Senar et al., 2000), and darker barn owl (*Tyto alba*) mothers produced offspring that develop a more symmetrical phenotype, mount a stronger humoral immune response against a non-pathogenic antigen, and are more resistant to ectoparasites (Roulin, 2004b).

Based on a literature review of genetic and pharmacological studies, Ducrest et al. (2008) for the first time proposed a genetic mechanism to explain how eumelanin-based coloration can be associated with resistance to stress in vertebrates. The most important regulators responsible for the synthesis of brown to black eumelanin pigments are the melanocortin 1 receptor (MC1R) and its ligands, the melanocortin agonists (MSHs and ACTH) and its antagonist, the agouti signaling protein (ASIP). Melanocortins are post-translational products of the *POMC*-gene and by binding to MC1R they trigger the production of eumelanin pigments, while binding of agouti-related protein blocks this production (Slominski et al., 2004). ACTH also stimulates the synthesis of glucocorticoids (cortisol and corticosterone) by binding to the melanocortin 2 receptor (MC2R) in adrenal glands (Simpson and Waterman, 1988). ACTH and glucocorticoids are hormones of the hypothalamic-pituitary-adrenal (HPA)-axis, which regulates the physiological stress response. Other melanocortins, in particular α-MSH, increase stress resistance by blocking the stress signal to the HPA-axis. Thus, Ducrest et al. (2008) proposed the hypothesis that in vertebrates in which inter-individual variation in the degree of eumelanin-based coloration is due to variation in melanocortin levels, dark individuals are more resistant to stress than lighter colored conspecifics.

To verify this hypothesis several types of studies are needed to show that the amount of melanocortins in different tissues is correlated with melanin-based coloration, and that melanocortins concomitantly affect the production of melanin pigments and resistance to stress. However, we first need to test experimentally that the degree of eumelanin-based coloration is indeed associated with resistance to stress. This experiment would provide not only a prerequisite basis for further studies investigating the role played by melanocortins in the resistance to stress, but also in two other respects. First, if melanocortins affect the resistance to stress, they also influence phenotypic plasticity to stressful rearing conditions. Stressful events lead to a glucocorticoid response (e.g. Kitaysky et al., 2001) and can strongly influence growth patterns which ultimately shapes the phenotype at adulthood (reviewed in Metcalfe and Monaghan, 2001). Hence, resistance to stress during growth is an important factor shaping the phenotype and ultimately fitness. Second, this experiment is important to better understand why melanin-based coloration is a mate choice criterion. In several species, both males and females select a mate based on the expression of melanin-based coloration as shown experimentally in the laboratory (Burley, 1977; Houtman and Falls, 1994; Fox et al., 2002) and as several field studies suggest (Roulin, 1999; West and Packer, 2002). Assuming that the degree of eumelanin-based coloration signals the ability to cope with

stressful environmental situations, darker eumelanic individuals may be selected as mates particularly in stressful habitats leading to the interesting possibility that mate choice is context-dependent (Roulin and Bize, 2007).

In the present study, we experimentally tested the hypothesis that darker eumelanic barn owls are more resistant to stress than less eumelanic conspecifics. To this end, we artificially administrated the mediator of stress responses in birds, corticosterone, in nestlings to examine the prediction that corticosterone-induced reduction in growth rate (Sapolsky et al., 2000; Hull et al., 2007; Dong et al., 2007) is more pronounced in lightly colored owlets than in darker nest mates. This is a powerful experimental design because it allows the manipulation of stress without applying a stressor such as lack of food, parasite outbreak or presence of a predator. We thus implanted a corticosterone-releasing pellet in half of the nestlings and in the other half we implanted a placebo pellet. The specific prediction to this experimental design is that the difference in growth rate between corticosterone- and placebo-implanted nestlings covaries with the degree of eumelanin-based coloration. Before testing this prediction, we verify two key assumptions, namely that implantation of a corticosterone-releasing pellet raises basal blood corticosterone level and does not influence the expression of eumelanin-based coloration.

Methods

Study site and study species

The study was carried out in an area of 190 km^2 in Western Switzerland (46°49'N, 06°56'E) at an altitude ranging from 430 to 520 m in 2004. The area is dominated by agriculture and holds a barn owl population of 20 - 80 pairs nesting in 110 nest-boxes put in barns (in 2004 we monitored 48 clutches). The nocturnal barn owl is a medium-sized predator of small mammals (99% of the diet, Roulin, 2004c). The two to eleven eggs are laid between March and July, and eggs hatch asynchronously on average every two to three days creating a pronounced within-brood age hierarchy. Maximal growth rate takes place at 17 days of age and at 40 days nestlings lose weight before fledging. Nestlings take their first flight at about 56 days of age and return to the nest until they reach 11-14 weeks of age before dispersing. Variation in plumage traits is already visible in nestlings, with females typically displaying a darker reddish-brown plumage than males (a phaeomelanin-based trait) and more and larger black spots (a eumelanin-based trait) than males, although individuals of both sexes can display any phenotype. Because in the barn owl resistance to stressful factors was found to correlate with the size of black spots and neither with number of spots nor with plumage coloration (Roulin et al., 2001; Roulin et al., 2003; Roulin et al., 2008; Almasi et al., 2008), we carried out statistical analyses only with the plumage trait 'spot diameter' to simplify

the analyses. Note however that if in the statistical analyses we replaced spot diameter with number of spots or plumage coloration, none of these two plumage traits were associated with growth in both the corticosterone and placebo treatments.

Mean spot diameter in sons is more strongly correlated with mean spot diameter of the biological mother than biological father and mean spot diameter of the daughters correlates with mean spot diameter of the father and only weakly with mean spot diameter of the mother. This pattern can be explained by a sex-linked inheritance of spot diameter, since in birds females are heterogametic (Roulin and Dijkstra, 2003). Cross-fostering experiments carried out with a large number of nests did not reveal any significant effect of environmental rearing conditions on spot diameter suggesting a strong genetic control (Roulin et al., 1998; Roulin, 2003).

Experimental design

To partition the effect of an experimental elevation of corticosterone level on growth into the origin-related and environmental components, we performed a partial cross-fostering experiment using 26 first annual broods. At hatching, broods were matched in 13 nest-box pairs (hereafter 'pair') with the criterion that nestlings were similarly aged. For each brood used in cross-fostering experiments two of the four oldest hatchlings (mean ± SD age in days: 29 ± 4) were randomly chosen and swapped between nests of the same pair so that each experimental nest contained nestlings of two origins. Nestlings were thus raised either by their biological parents in their nest of origin or in the nest of foster parents referred to as 'nest of rearing'. Thus, for individuals raised by biological parents the nests of origin and rearing were the same, while for individuals raised by foster parents the nest of rearing and of origin were different.

To investigate the effect of increased plasma levels of corticosterone on nestling growth we implanted the 4 oldest nestlings (mean age ± SD at implantation: 29 ± 4 days) with either a corticosterone or a placebo implant. The implants (diameter 5 mm) are made up of a biodegradable carrier-binder containing 15 mg corticosterone or, for placebo, only of the biodegradable carrier-binder (Innovative Research of America, Sarasota, Florida). We implanted the pellet under the skin of the flank above the knee through a small incision, which was closed with tissue adhesive (Histoacryl, Braun, Germany). The implants were specified to have a given constant release rate of 7 days in rats. One of the cross-fostered individuals and one of the non-cross-fostered nest mates were implanted with a corticosterone pellet (hereafter cort-nestlings) and the other cross-fostered individual and non-cross-fostered nest mate with a placebo pellet (hereafter placebo-nestlings). In nine broods only two nestlings survived until the start of the experiment and we implanted one nestling with corticosterone implants and one with a placebo implant. In total 43 nestlings received

a corticosterone implant and 43 other nestlings a placebo implant (31 nestlings raised in the 26 experimental nests were not implanted). At the day of implantation cort- and placebo-nestlings did not differ with respect to age (Student's t-test, $t_{74} = 0.71$, $p = 0.48$), body mass (324 ± 46 g (SD), $t_{74} = 0.62$, $p = 0.54$), and wing-length (129 ± 25 mm (SD), $t_{73} = 0.69$, $p = 0.49$). We allocated as many female as male nestlings in the two treatments (χ^2-test, $\chi^2 = 0.191$, $p = 0.662$).

Plasma corticosterone

To monitor the effect of the implants on circulating total corticosterone we collected blood samples just before implantation, 2, 6 and 20 days after implantation, by puncturing the brachial vein and collecting the blood with heparinised capillary tubes. Samples were immediately centrifuged and the plasma stored in liquid nitrogen. After transport to the laboratory, the samples were stored at –20°C until analysis in the next autumn. Since nestlings started to increase plasma corticosterone levels 3 minutes after having their nest-box opened (personal observation), we used only blood samples taken within 3 minutes of first opening the nest-box as baseline corticosterone level for analysis. Plasma corticosterone concentration was determined using an enzyme immunoassay (Munro and Stabenfeldt, 1984; Munro and Lasley, 1988). For details on the assay see Müller et al. (2006).

Assessment of nestling growth, plumage traits and gender

To investigate the effect of corticosterone administration on growth, we weighed all 86 nestlings to the nearest 0.1 g, measured maximum wing length and tarsus length to the nearest mm on the day of implantation, as well as 2, 6, 14 and 20 days after implantation.

We recorded plumage traits in all nestlings just before fledging and in breeding adults. A. R. took these measurements blind to treatments because at that time only B. A. knew which individuals were implanted with a corticosterone or a placebo pellet. We compared phaeomelanin-based coloration of breast, belly, one flank and one underside of the wings with eight color chips, ranging from 1 for reddish-brown to 8 for white. To assess the variation in number and size of black spots, we placed a 60x40 mm frame on the same four body parts where we counted the number of spots and measured their diameters to the nearest 0.1 mm. For each body part we calculated a mean spot diameter. For each bird we then calculated a mean coloration, spot diameter and spot number using measures taken on the four body parts. For details on the methods of assessing plumage traits and their reliability see Roulin and Dijkstra (2003) and Roulin (2004b).

The sex of all nestlings was determined using the CHD-gene method (for details on the method see Py et al., 2006). Breeding females were recognized by the presence of a brood patch.

Statistical procedure

The growth-curves of nestling body mass, wing and tarsus lengths were analysed with mixed-effect models with the two random factors 'nest of origin' and 'nest of rearing' both nested in 'pair' plus their interactions with the fixed factor 'implantation' (i.e. corticosterone vs. placebo). To keep the random model as simple as possible the term 'pair' was removed when it did not explain the variance. Since implantation of corticosterone-pellets caused a decline in body mass in the first two days after implantation but not thereafter (see results), we performed a mixed-effect-model analysis on the change in body mass from day 0 to day 2 of the experiment (hereafter 'initial mass change') with 'implantation' and 'sex' as two categorical variables, and nestling age at the start of the experiment ('age'), and brood size as covariates. We built the models with all possible interactions and compared it with a one term simpler model. Models were compared with the log likelihood ratio test (LRT) and the more complicated model was kept when it was significantly better than the simpler model, otherwise the simpler model was kept. We always included 'age' into the model to correct for both the pronounced age hierarchy in barn owl broods, which results in body mass differences between nest mates, but also for variation in body condition at the start of the experiment. With the best model we made then two separate analyses. In the first analysis we included 'spot diameter' of both, the biological mother and father, plus their interaction with 'implant'. Using a similar model selection procedure we reduced the model so that it contained only significant variables. In the second analysis we included 'spot diameter of the nestlings' instead of the parental spot diameters and proceeded in the same way. All random and fixed effects of the final models were tested using a 'Monte Carlo simulation' approach after Faraway (2006). Thereby, the distribution of the likelihood ratio for comparing an alternative model (containing term X) with a null model (model without term X) is approximated using Monte Carlo simulation. We simulated 1'000 times a set of response values from the null model and calculated the likelihood ratio between the alternative and the null model for each set of simulated response values. From these 1'000 likelihood ratios an approximation of the distribution of the likelihood ratio can be obtained which is more appropriate than the Chi-square distribution (Faraway 2006).

To describe body mass gain from day 2 to day 20 after the start of the experiment we calculated the slope of the growth curve of each nestling with the following formula: $y = \beta * d + i$, where y is body mass of the nestlings, d the day after start of the experiment (2, 6, 14, 20 days after start of the experiment), β the slope (hereafter secondary mass gain), and i the intercept. Then we performed the same mixed-effect model analysis as described above with the secondary mass gain of each nestling as dependent variable. To describe the wing and tarsus growth curves we calculated the slope and quadratic effect of each nestling's growth curve using the following formula:

$y=\beta_1*d+\beta_2*(v\text{^}2)$, where y is either wing length (or tarsus length) minus wing length (or tarsus length) at the start of the experiment, d is the number of days after the start of the experiment (0, 2, 6, 14, 20 days), β_1 the linear effect (hereafter linear term) and β_2 the quadratic effect (hereafter quadratic term). We subtracted wing length (or tarsus length) measured on day 0 of the experiment from the other measurements taken at day 2, 6, 14, and 20 of the experiment. We analysed linear wing and tarsus growth and the quadratic wing and tarsus growth separately using the same mixed-effect model analysis as described above. Finally, to see whether corticosterone implantation had a long-term effect on nestling body size, we analysed body mass, wing length, and tarsus length measured shortly before fledging (at the age of 49 ± 3.9 days (SD) 20 days after the start of the experiment) using the same mixed-effect model analysis as described above.

All statistical tests were done using the statistical software package R version 2.4.1 (R Development Core Team, 2006). Means are quoted ± SE if not indicated otherwise. P-values < 0.05 were considered as significant.

Ethical note

Nestling survival was not affected by corticosterone administration. In the 26 broods a total number of 117 nestlings hatched and 108 survived until fledging (92%), a value comparable to a previous non-experimental study in 2005 where 159 of 179 nestlings survived until fledging (88.2%). Of these 117 nestlings 86 either obtained a corticosterone or a placebo implant and 81 of them survived until fledging (three cort- and two placebo-nestlings died before fledging). The study was done under legal authorization of the 'Service vétérinaire du canton de Vaud', permit n° 1736.

Results

Corticosterone implantation: effectiveness and effect on spot diameter

Corticosterone implantation significantly increased corticosterone concentration above baseline level (mixed-effect model with 'nestling identity' nested in 'nest of rearing' as random factor; implant × days after implantation: LRT = 4.14, p = 0.042). Post-hoc tests showed that there was no difference in corticosterone level between the two treatments at the day of implantation (post-hoc mixed-effect model with nest of rearing as random factor: LRT = 0.30, p = 0.582). This demonstrates that we allocated nestlings to the two treatments randomly with respect to baseline corticosterone level. Two days after corticosterone implantation cort-nestlings had a significantly elevated corticosterone level (similar model: LRT = 24.73, p < 0.001), and 20 days after

implantation there was no differences between the treatments anymore (similar model: LRT = 0.51, p = 0.476; Fig. 1).

Corticosterone implantation did not affect significantly nestling spot diameter (mixed-effect model with 'nest of rearing' as random factor, implant: LRT = 1.65, p = 0.200; mean spot diameter of cort-nestlings: 14 ± 0.71 mm, placebo-nestling 14 ± 0.66 mm).

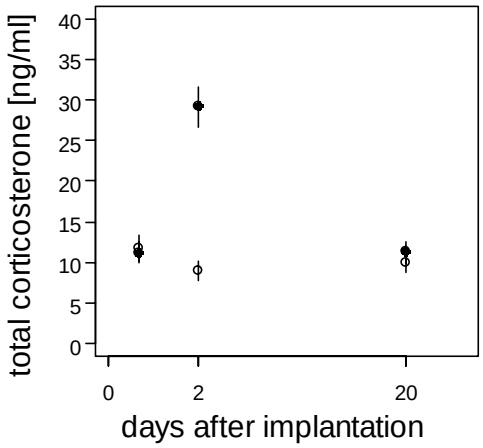

Fig 1. Mean total corticosterone ± SE at the day of implantation, 2 and 20 days after implantation of cort-nestlings (closed symbols) and placebo-nestlings (open symbols).

Body mass

Corticosterone implantation significantly reduced body mass during the first two days post-implantation compared to placebo-nestlings (Table 1A). The random effect 'nest of rearing' but not 'nest of origin' explained a significant proportion of the variance of initial mass change. Between day 2 and day 20 (the period of secondary mass gain), cort-nestlings gained significantly more body mass than placebo-nestlings (effect size of corticosterone implants: 1.17 ± 0.31 g d^{-1}, Fig. 2) indicating compensatory growth in cort-nestlings. The age at the start of the experiment had also a significant effect on secondary mass gain: a one day older nestling gained 0.40 ± 0.04 g d^{-1} less than a one day younger nestling, but not in interaction with the implantation. 'Nest of origin' and 'nest of rearing' had no significant influence on secondary mass gain.

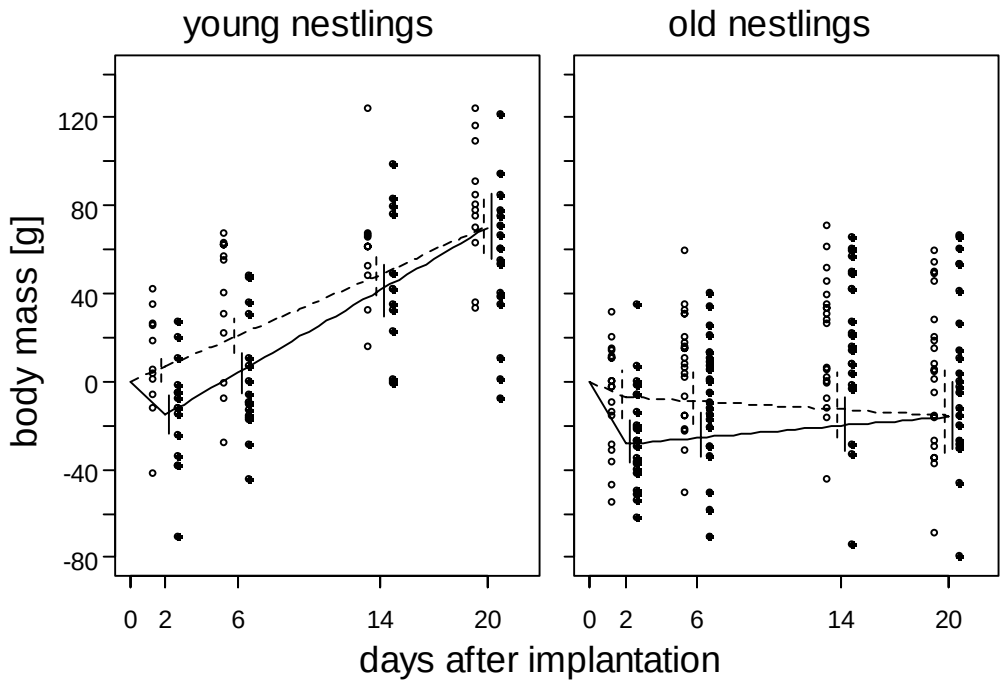

Fig 2. Body mass change from day 0 to day 20 of the experiment of young nestlings and old nestlings. Individuals implanted at an age below the median (28.8 days) were denoted 'young individuals' and those implanted above the median 'old individuals'. Closed symbols represent cort-nestlings, open symbols placebo-nestlings. Lines represent the predicted mass and confidence intervals of the full model (see Table 1 A) of cort- (line) and placebo-nestlings (dashed line). All birds start at a body mass of 0 at day 0.

As shown by the significant interaction between implantation and nestling spot diameter, fledging body mass was differentially affected by corticosterone depending on nestling spot diameter (Table 1A). Cort-nestlings with small spots were lighter in body mass than cort-nestlings with large spots, while body mass was not associated with spot diameter in placebo-nestlings (Fig. 3). Age at implantation had no significant effect on fledging body mass. Female nestlings were significantly heavier than male nestlings. 'Nest of origin' and 'nest of rearing' had no significant influence on fledging body mass.

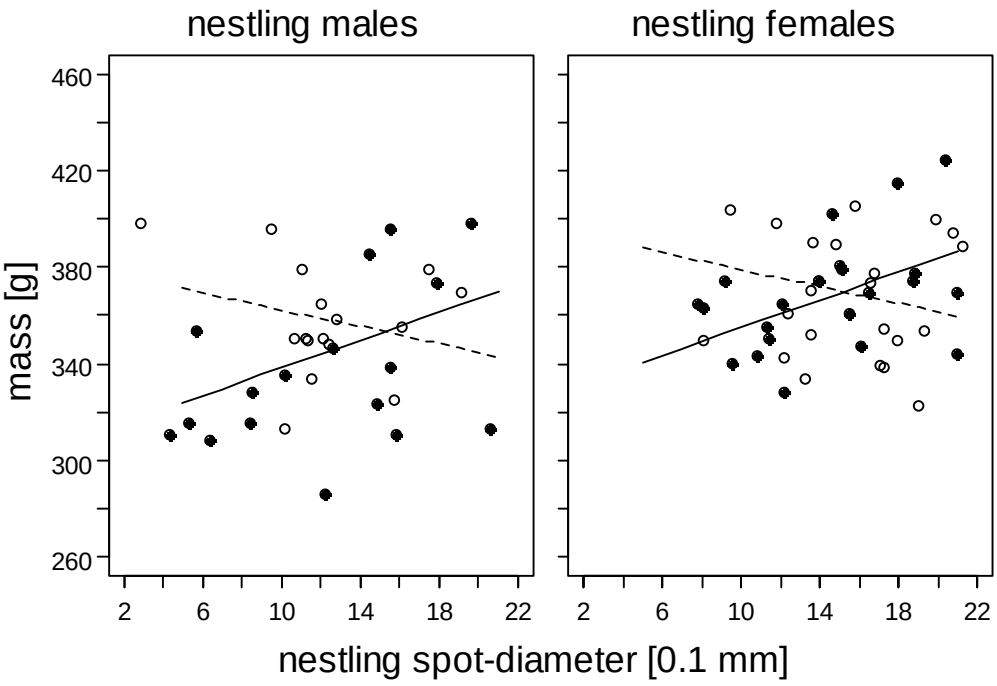

Fig 3. Body mass shortly before fledging of male nestlings and female nestlings. Closed symbols represent cort-nestlings, open symbols placebo-nestlings. Lines represent the predicted mass of the full model (see Table 1A) of cort- (line) and placebo-nestlings (dashed line). Post hoc test showed that cort-nestlings with large spots were significantly heavier than cort-nestlings with small spots (mixed-effect model with 'nest of rearing' and 'nest of origin' as random factor, LRT = 6.81, p = 0.009), whereas placebo-nestlings had the same body mass regardless of spot diameter (mixed-effect model with 'nest of rearing' and 'nest of origin' as random factor, LRT = 1.43, p = 0.232).

Wing length

Corticosterone implantation reduced wing length growth differentially depending on spot diameter of the nestlings. This effect was significant in the linear and quadratic term of the wing length growth curve (Table 1B). Nestlings with large spots were less affected by the corticosterone implantation than nestlings with small spots (Fig. 4). The age of the nestlings at the start of the experiment had a significant negative effect on the linear term (effect size of age: -0.1 ± 0.02 mm d^{-1}) but explained no significant proportion of the variance of the quadratic term. 'Nest of rearing' explained a small significant proportion of the variance of the linear and the quadratic term. 'Nest of origin' had no significant effect on wing-length growth.

Wing length at fledging was significantly shorter in cort-nestlings (effect size of cort-implantation: -5.1 ± 1.0 mm d^{-1}) than in placebo-nestlings. Age at the start of the experiment (effect

size of age: 4.5 ± 0.23 mm d^{-1}) had a significant positive influence on fledging wing length and females had significantly longer wings than males (effect size of sex: 4.8 ± 1.2 mm d^{-1}). Neither 'nest of origin' nor 'nest of rearing' explained a significant proportion of the variance of fledging wing length.

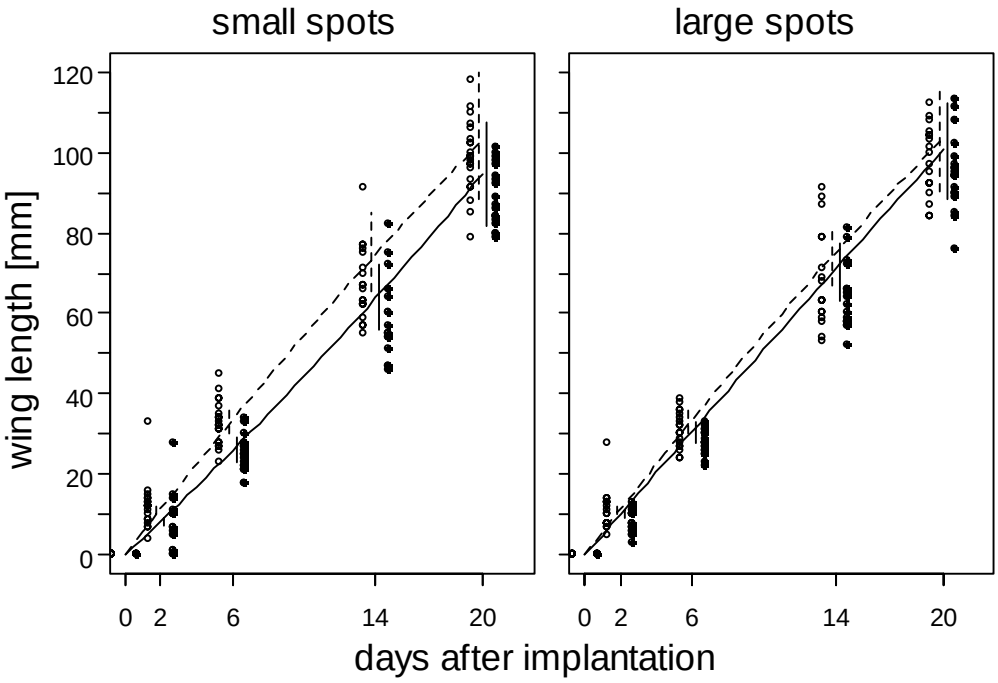

Fig 4. Wing length growth from day 0 to day 20 of the experiment of nestlings with small spots and nestlings with larger spots. Individuals with spot diameter below the median (1.44 mm) were denoted 'small spots' and those nestlings with spot diameter above the median 'large spots'. Closed symbols represent cort-nestlings and open symbols placebo-nestlings. Lines represent the predicted wing length and confidence intervals of the full model (see Table 1 B) of cort- (line) and placebo-nestlings (dashed line). For all measurements we subtracted wing length measured at day 0. Post-hoc tests showed that wings of large-spotted cort-nestlings grew more rapidly than wings of small-spotted cort-nestlings (mixed-effect model with 'nest of rearing' and 'nest of origin' as random factor, linear term: LRT = 3.86, p = 0.045, quadratic term: LRT = 0.19, p = 0.661), whereas wing growth of small- and large-spotted placebo-nestlings was similar (mixed-effect model with 'nest of rearing' and 'nest of origin' as random factor, linear term: LRT = 9.39, p = 0.002, quadratic term: LRT = 0.04, p = 0.839).

Tarsus length

Tarsus growth was significantly affected by cort-implantation, but differently according to age, an effect that was present in the linear and quadratic term of the tarsus growth curve (Table 1C, Fig. 5).

Neither 'nest of origin' nor 'nest of rearing' explained a significant proportion of the variance of the tarsus growth curve.

Tarsus length at fledging was not affected by cort-implantation, but there was a small significant influence of age at the start of the experiment on fledging tarsus length (effect size of age: 1.6 ± 0.7 mm d^{-1}) suggesting that the smallest nestlings still continued to grow after the last measurement. Neither 'nest of origin' nor 'nest of rearing' explained a significant proportion of the variance of fledging tarsus length.

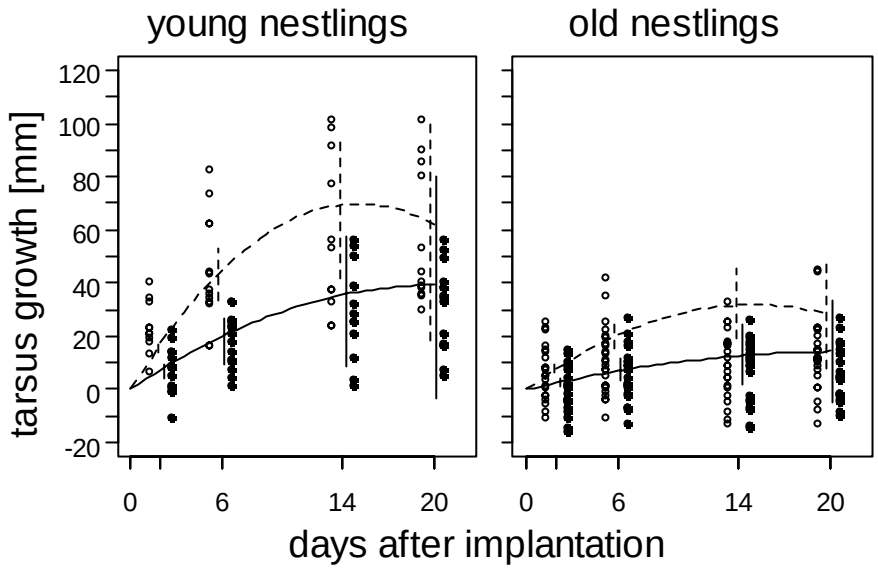

Fig 5. Tarsus growth from day 0 to day 20 of the experiment of young and old nestlings. Individuals implanted at an age below the median (28.8 days) were denoted 'young individuals' and those implanted above the median 'old individuals'. Closed symbols represent cort-nestlings and open symbols placebo-nestlings. Lines represent predicted tarsus length and confidence intervals of the full model (see Table 1C) of cort- (line) and placebo-nestlings (dashed line). For all measurements we subtracted tarsus length measured at day 0. Negative values are due to measurement errors. Post-hoc tests showed that corticosterone treatment significantly affected tarsus growth in young nestlings (mixed-effect model with 'nest of rearing' and 'nest of origin' as random factors, linear term: LRT = 20.80, $p < 0.001$, quadratic term: LRT = 17.13, $p < 0.001$), there was no significant effect in old nestlings (mixed-effect model with 'nest of rearing' and 'nest of origin' as random factors, linear term: LRT = 3.54, $p = 0.060$, quadratic term: LRT = 3.09, $p = 0.079$).

Table 1. Results of the mixed-effect model analysis with A. body mass growth ('initial mass change' (mass change from day 0 to day 2 of the experiment), 'secondary mass gain' (slope of the growth curve from day 2 to day 20 of the experiment), and 'fledging body mass), B. wing length growth ('linear term' (linear effect of the wing length growth curve), 'quadratic term' (quadratic effect of the wing length growth curve), and 'fledging wing length'), and C. tarsus growth 'linear term' (linear effect of the tarsus length growth curve), 'quadratic term' (quadratic effect of the tarsus length growth curve), and 'fledging tarsus length') as dependent variables. Shown are degree of freedom (df), log-likelihood ratio test (LR), and the p-values obtained with the bootstrap method (Faraway 2006). Measurements were taken on 43 cort- and 43 placebo-nestlings on 5 different days during a period of 20 days. Where indicated with asterisk the random effects are: *: 'Pair:Origin' and 'Pair:Rearing'; ** 'Origin' and 'Rearing'.

	df	LR	p_{boot}	df	LR	p_{boot}	df	LR	p_{boot}
A. body mass	**initial mass change**			**secondary mass gain**			**fledging body mass***		
Random effects									
Origin	1	0.00	0.730	1	2.45	0.052	1	0.78	0.163
Rearing	1	6.32	**0.003**	1	2.19	0.056	1	0.00	0.471
Fixed effects									
Sex							1	8.50	**0.005**
Age	1	3.11	0.083	1	55.32	**<0.0001**	1	1.62	0.255
Implant	1	17.03	**<0.0001**	1	12.63	**<0.0001**	1	0.64	0.470
Dia							1	1.09	0.327
Implant*Dia							1	11.31	**<0.0001**
B. wing	**linear term**			**quadratic term****			**fledging wing length**		
Random effects									
Pair:Origin	1	0.00	0.689	1	0.00	0.975	1	0.00	0.715
Pair:Rearing	1	27.43	**<0.0001**	1	43.35	**<0.0001**	1	8.14	0.458
Fixed effects									
Sex							1	14.01	**<0.0001**
Age	1	19.70	**<0.0001**	1	2.42	0.154	1	174.82	**<0.0001**
Implant	1	33.15	**<0.0001**	1	25.54	**<0.0001**	1	19.96	**<0.0001**
Dia	1	5.96	**0.022**	1	3.72	0.056			
Implant*Dia	1	7.17	**0.013**	1	4.79	**0.045**			
C. tarsus	**linear term**			**quadratic term**			**fledging tarsus length**		
Random effects									
Origin	1	0.02	0.310	1	0.00	0.391	1	0.41	0.179
Rearing	1	0.00	0.973	1	0.00	0.842	1	0.00	0.380
Fixed effects									
Age	1	79.85	**<0.0001**	1	47.09	**<0.0001**	1	4.98	**0.032**
Implant	1	28.35	**<0.0001**	1	20.57	**<0.0001**			
Implant*Age	1	11.87	**<0.0001**	1	7.27	**0.011**			

Discussion

In this study we demonstrated that an elevation of corticosterone level during a few days reduces growth temporarily in barn owl nestlings. The individual sensitivity to corticosterone administration measured in terms of growth reduction varied with spot diameter, an eumelanin-based trait. Under corticosterone administration, nestlings with larger black spots grew better than nestlings with smaller black spots.

Effect of corticosterone administration on growth

Implantation of corticosterone-releasing pellets led to a moderate increase in corticosterone level within the physiological range comparable to situations when nestlings were poorly fed (own personal observation). Artificial administration of corticosterone had a strong influence on all measured growth parameters. In our analysis the interactions 'nest of rearing by implant' were never significant which suggests that the rearing environment has no large impact on the resistance to stress. The rationale of experimentally elevating corticosterone level was to simulate a physiological stress response without applying a stressor, such as commonly applied with experiments that manipulate brood size, food supply, or challenge nestlings with an immune elicitor. With our design we could thus examine the individual abilities to cope with a physiological stress situation.

Adverse environmental conditions have immediate effects on nestling development and fitness (reviewed in Lindström, 1999). Previous studies in birds and mammals showed that food deprivation (Lynn et al., 2003) and low body condition (Wingfield et al., 1994; Schoech et al., 1997; Kitaysky et al., 1999; Cabezas et al., 2007) induce an elevation in corticosterone levels. In altricial bird species the HPA-axis starts to develop in the first days post hatching (Sims and Holberton, 2000; Wada et al., 2007; B. Almasi unpublished results) as shown by the observation that young nestlings are able to increase circulating corticosterone levels as a reaction to an acute stressor. At the start of the experiment, 29-day-old barn owl nestlings already showed a strong increase in corticosterone levels due to capture and handling stress (C. Müller et al., in preparation). Glucocorticoids directly interfere with the growth hormone-IGF-1 axis. An excess of glucocorticoids indeed reduces growth hormone secretion, decreases bone formation, and inhibits IGF-1 signaling, which leads to catabolic and anti-anabolic effects on muscle proteins (Hochberg, 2002). As a consequence, an artificial increase in corticosterone level should lead to a decrease in growth rate as already observed in birds and mammals (Sapolsky et al., 2000; Huang et al., 2000; Hull et al., 2007; Dong et al., 2007). Accordingly, in nestling barn owls corticosterone implantation reduced body mass two days after implantation significantly compared to placebo implantation. Body mass of younger nestlings was reduced to a larger extent than in older nestlings. Once the effect of the corticosterone-releasing pellets on circulating corticosterone level started to cease three days after implantation (unpublished data), cort-nestlings accelerated body-mass gain compared to placebo-nestlings indicating a catch-up growth strategy. Although body mass at fledgling did not differ between cort- and placebo-nestlings (Table 1), there was a significant interaction between nestling spot diameter and treatment, a finding that we discuss below.

Wing length growth was reduced in nestlings with artificially elevated corticosterone levels. The quadratic term of the wing length growth curve of cort-nestlings was slightly smaller than that of placebo nestlings, suggesting that wing length growth of cort-nestlings is prolonged compared to placebo-nestlings indicating a catch-up growth strategy as found for body mass. However, contrary to body mass, wing length at fledging was still smaller in cort-nestlings than wing length in placebo-nestlings indicating that catch-up growth takes more time for wing length than body mass. This is consistent with a study in Alpine swift (*Apus melba*) showing that after a temporary period of food shortage nestlings catch-up in growth more rapidly with respect to body mass than wing length (Bize et al., 2006). Apparently, body mass has a higher priority of tissue preservation compared to wing length (see also Bize et al., 2003; Alvarez and Nicieza, 2005; Bize et al., 2006) which still continue to grow after fledging in the barn owl. Therefore, it is still possible that wing length is fully compensated for during the first weeks post-fledging by extending the period of growth. Tarsus growth of cort-nestlings was reduced compared to placebo-nestlings, and again nestlings appeared to catch-up growth as at fledging tarsus length was no more different between treatments. Other studies in birds and mammals found that elevated corticosterone levels and food restriction reduced tarsus growth (Boag, 1987; Baron et al., 1994; Searcy et al., 2004). Assuming that skeletal size is important for sibling competition within the nest and for hunting success after fledging, selection may be strong on the ability to do catch-up growth in the barn owl. From a proximate point of view, catch-up growth may be possible because barn owls show an overshoot in body mass at 40 days possibly allowing them to buffer stressful situations occurring before that age as it was the case with our experiment. The hypothesis that body mass increase is an adaptation to circumvent the negative impact of poor rearing conditions has been suggested by Lack (1968). However, a recent study showed that during body-mass recession nestlings loose mainly body water, whereas lipid reserves still increase (Phillips and Hamer, 1999). Also the body mass increase during laying in breeding barn owl females is not a consequence of lipid but mainly of water accumulation. The water accumulation is suggested to be the consequence of an increased protein metabolism (Durant et al., 2008). Thus the energy safety strategy to explain the body mass increase in nestlings and breeding females has to be reconsidered. However, if cort-nestlings do not have the same recession in body mass than placebo nestlings and therefore do not loose body water, cort-nestlings might still have lower lipid reserves at fledging than placebo-nestlings.

Corticosterone-mediated reduction in growth rate in relation to the degree of eumelanin-based coloration

Often it remains unknown whether phenotypic variation emerges from differences in the genotype or rearing conditions (environmental influences or parental care). Here we showed that corticosterone administration during development differentially affected nestling growth depending on the degree of eumelanin-based coloration of the nestlings, a heritable trait for which the expression is not sensitive to rearing conditions (Roulin and Dijkstra, 2003). Using a cross-fostering experimental design we allocated nestlings randomly among habitats with respect to spot diameter, and hence the relationship between spot diameter and resistance to stress measured in terms of growth rate suggests a genetic effect. The interactions 'nest of origin by implant' were also not significant which leads to two different interpretations. First, the ability to cope with stress, which is signaled in spot diameter, is under genetic control but spot diameter of nestlings from one brood pair varies to a comparable level as between nestlings from different nests. This hypothesis may explain why we did not detect a significant interaction of 'nest of origin by implant' but of spot diameter. Second, the ability to resist stress is not genetically determined and the significant effect of spot diameter might be due to within-nest behavioral differences between nestlings that are associated with spot diameter and determines resistance to stress. This possibility is unlikely because it would predict that heavily spotted nestlings should perform better in both the corticosterone- and placebo-treatments, which was not the case. As figure 3 shows, the relationship between body mass growth and spot diameter was significant only in the corticosterone-treatment. Thus, the finding that a short-term elevation of corticosterone levels affects growth rate differently in heavily- and lightly-spotted nestlings suggests that the ability to cope with stress has a genetic component, which is associated with the degree of eumelanin-based coloration. Thus, the reaction norms of heavily- and lightly-spotted individuals are different according to the level of stress incurred during the rearing period. This is new evidence that developmental plasticity in response to variation in the rearing environment is associated with the degree of melanin-based coloration (Roulin et al., 2008).

In the barn owl, it has been shown that males mate non-randomly with respect to the size of black spots displayed by females, and they invest more effort in reproduction when their partners display larger black spots (Roulin, 1999). Males may pair preferentially with females displaying larger black spots because this trait is a heritable signal of quality with females displaying larger spots showing a higher survival (Roulin and Altwegg, 2007) and producing offspring that are more immunocompetent, resistant to parasites and developmentally more stable (Roulin, 2004b). The present study adds new information on the signaling function of eumelanin-based coloration in the

barn owl, as dark nestlings were significantly better able to cope with an experimental elevation in corticosterone levels than lightly colored nestlings. As stated in the introduction, the link between the degree of eumelanin-based coloration and resistance to stress may not be restricted to the barn owl as it might be explained by the genetic mechanism underlying the production of eumelanin pigments.

Implications and perspectives

Our experimental design created two different rearing conditions (stressful vs. relaxed) for nestlings in each single nest. With this design we were able to look at the impact of environmental heterogeneity on nestling development in relation to a sexually selected trait. Our results showed that in stressful rearing conditions darker eumelanic individuals have an advantage in terms of growth, potentially indicating that the degree of eumelanin-based coloration may be directionally selected in stressful environments as already suggested in another study (Roulin et al., 2008). This raises the question of how genetic variation in eumelanin-based coloration is maintained within the barn owl populations and what is the benefit of displaying small eumelanic spots. So far in the barn owl only advantages of displaying large eumelanic spots have been detected (e.g. Roulin et al., 2001; Roulin, 2004b; Roulin and Altwegg, 2007) while no evidence for an advantage of not being lightly eumelanic could be yet found. This suggests that other mechanisms should be involved to explain the maintenance of genetic variation in the size of eumelanic spots. This possibility is currently being analysed using long-term datasets.

As mentioned in the introduction, the hypothesis suggested by Ducrest et al. (2008) proposes that in vertebrates in which inter-individual variation in the degree of eumelanin-based coloration is due to variation in melanocortin levels, dark individuals are more resistant to stress than lighter colored conspecifics because melanocortins are directly involved in resistance to stress. Our results and results from previous studies (Roulin et al., 2008; Almasi et al., 2008) are consistent with this hypothesis which provides a promising avenue for future studies. A next key step will be to firmly demonstrate the role of melanocortins in generating covariation between the degree of eumelanin-based coloration and resistance to stress. To this end, we will first have to demonstrate that the amount of melanocortins in different tissues is correlated with melanin-based coloration, and then to show that melanocortins concomitantly affect the production of melanin pigments and resistance to stress.

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Chapter 4

Stress sensitivity is heritable and signalled in eumelanin-based coloration

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Abstract

Heritable phenotypic traits, which correlate with fitness-relevant traits, can be chosen by sexual selection. Stress sensitivity might be such a fitness-relevant trait. As a reaction to a stress situation a physiological stress response takes place, which includes the release of stress hormones. The main stress hormones in vertebrates are glucocorticoids, which control the physiological and behavioural responses to overcome a stress situation, but can entail cost such as a higher risk of infection and reduced resistance against oxidative stress. Thus, animals need to regulate their glucocorticoid secretion in tight response to the stress situation. In the present study we investigated whether stress sensitivity is a heritable trait, which is signalled in a parental melanin-based coloration. We experimentally elevated corticosterone levels in barn owl nestlings and looked whether we could explain the variation in circulating corticosterone level regulation due to melanin-based coloration of the parents. We found that corticosterone levels of nestlings of more eumelanic mothers are lower after corticosterone administration than corticosterone levels of nestlings of less euemalnic mothers. This result suggests that nestlings from more eumelanic mothers have a more sensitive regulation of circulating corticosterone levels possibly through a more sensitive negative feedback mechanism.

Introduction

Selection acts on individual variation in morphology, behaviour and physiology, but the endocrinological basis of individual variation and how it contributes to variation in fitness is still poorly known (reviewed in Zera et al., 2007). It is well known that traits often covary. Sexual selection can operate when a phenotypic trait (e.g. song or coloration) covaries with a fitness-relevant trait. For instance individual differences in endocrinological responses can be signalled in phenotypic traits (Wada et al., 2008), and can therefore play a role in mate choice. In many amphibian and bird species, females choose males according to their call and song performance. Females of the Great Plains toad (*Bufo cognatus*) prefer males with longer call durations, and males which perform longer calls had lower corticosterone levels than males with shorter call duration (Leary et al., 2006). Zebra finch (*Taeniopygia guttata*) females prefer males with long song durations and a high complexity of the song performance, and elevated corticosterone levels during development reduce song duration and complexity (Spencer et al., 2003). That coloration plays a role in mate choice and can honestly signal an individual's quality has been studied in different species (e.g. Houtman and Falls, 1994; West and Packer, 2002; Fox et al., 2002), but that coloration shows an individual's ability to cope with stressful situations has been proposed only recently. Female zebra finches for example choose males which were selected for low peak corticosterone levels over males selected for higher peak corticosterone levels and males from the low peak corticosterone line show different leg, beak and cheek brightness, which are sexual selected phenotypic traits, compared to the high corticosterone males (Roberts et al., 2007).

Little is known about the mechanisms explaining why physiological traits covary with other traits. Hormones often influence multiple traits (hormonal pleiotropy) (reviewed in Zera et al., 2007) and thus may lead to covariation between traits. In this context, sensitivity to stress or coping with stress is an important trait that may be related to many other traits through glucocorticoids, the main stress hormones. Glucocorticoids, which include corticosterone and cortisol, are hormones of the hypothalamic-pituitary-adrenal (HPA)-axis and control the physiological and behavioural responses to overcome a stress situation. The actions of glucocorticoids include, amongst others, elevated arterial pressure and heart rate, reallocation of stored energy, increased food searching behaviour and suppression of immune functions and reproduction (Wingfield et al., 1998; Sapolsky et al., 2000; Romero, 2004). Glucocorticoids further affect development, thus shape the phenotype at adulthood in many respects (Hull et al., 2007; Dong et al., 2007). The actions of glucocorticoids are well studied, but little attention was given to individual differences in the glucocorticoid response and its consequence for individual fitness and genetic variation of populations. An important new hypothesis, based on a functional link between melanogenesis and the HPA-axis,

proposes that in vertebrates in which inter-individual variation in the degree of eumelanin-based coloration is due to variation in melanocortin levels, dark individuals are more resistant to stress than lighter coloured conspecifics (Ducrest et al., 2008). This opens the possibility that stress sensitivity is directly linked to coloration, which often is a criterion of sexual selection.

Support for this hypothesis comes from barn owls (*Tyto alba*) and tawny owls (*Strix aluco*), whose offspring of parents with more phaeomelanin-based coloration grew more rapidly in body mass in more relaxed conditions (experimentally reduced brood-size) and Alpine swift (*Apus melba*) offspring of more eumelanic fathers grew their wings more rapidly in more stressful environmental conditions (experimentally enlarged broods) (Roulin et al., 2008). A further study showed that breeding barn owl males showed plasticity in parental investment as a response to experimentally elevated corticosterone levels: males with less eumelanin-based coloration provisioned more food to their brood than more eumelanic males, but when they had elevated corticosterone levels they reduced their provisioning rate much more than more eumelanic males (Almasi et al., 2008). Furthermore, elevated corticosterone levels reduced nestling growth more in less eumelanic nestlings than it reduced growth in more eumelanic nestlings (Almasi et al., in preparation).

Different mechanisms are possible to explain the differences in the response to elevated corticosterone levels. One possibility is that less eumelanic individuals are more stress sensitive, i.e. they have a stronger behavioural or developmental response to a given elevated corticosterone level. Another possibility is that the HPA-axis of more eumelanic individuals is better able to regulate circulating corticosterone levels. The mechanism behind a differential regulation of circulating corticosterone levels associated with eumelanin-based coloration may be the pleiotropic effects of melanocortins which regulate melanogenesis and other physiological functions such as homeostasis and stress sensitivity (Ducrest et al., 2008). If stress sensitivity is signalled in eumelanin-based coloration, it is important to know whether stress sensitivity is heritable and thus may come under sexual selection.

In this study, we investigated in nestling barn owls whether stress sensitivity is signalled in plumage melanism and whether it is a heritable trait, i.e. that stress sensitivity is signalled in parental plumage melanism. We chose the barn owl as a model species since a heritable melanin-based female ornament is used as a male mate choice criterion (Roulin, 1999; Roulin, 2004; Roulin and Altwegg, 2007) and previous experimental studies have shown that this plumage trait is associated with the ability to cope with stress (Roulin et al., 2003; Almasi et al., 2008). Within one brood, we created two stress levels by implanting the stress hormone corticosterone in half of the

experimental nestlings. The subsequent increase in plasma corticosterone level is taken as a measure of stress sensitivity. We cross-fostered the nestlings to disentangle environmental from genetic factors. If stress sensitivity is at least partly heritable and signalled in plumage melanism, we predict that nestlings with more eumelanic parents are better able to regulate circulating corticosterone levels after artificial corticosterone administration.

Methods

Study design and cross-fostering

The study was carried out on a plain covering 190 km^2 at an altitude ranging from 430 to 520 m in Western Switzerland (46°49'N, 06°56'E) in 2004 and 2006. The area is dominated by agriculture and holds a barn owl population of 20 - 80 pairs nesting in 110 nest boxes put in barns.

At hatching, broods were matched in nest-box pairs with the criterion that nestlings were similarly aged. In 2004 two of the four first hatched nestlings were randomly chosen and in 2006 the four first hatched nestlings were chosen and swapped between nest-box pairs. To investigate the effect of increased plasma levels of circulating corticosterone we implanted the four oldest nestlings from 23 experimental broods in 2004 and 19 experimental broods in 2006 with either a corticosterone or with a placebo implant, so that each nest contained two corticosterone-implanted nestlings (hereafter cort-nestlings) and two placebo-implanted nestling (hereafter placebo-nestlings). In this study we only considered nestlings raised by foster parents, thus one cort-implanted and one placebo-implanted nestling in the 2004 broods and two of each in the 2006 broods.

The implants (diameter 5 mm) are made up of a biodegradable carrier-binder containing 15 mg corticosterone or, for placebo, only of the biodegradable carrier-binder (Innovative Research of America, Sarasota, Florida). We implanted the pellet under the skin of the flank above the knee through a small incision, which was closed with tissue adhesive (Histoacryl, Braun, Germany). The implants were specified to have a given constant release rate of seven days. Fifty-eight nestlings received a corticosterone implant (22 nestlings in 2004 and 36 in 2006) and 54 nestlings a placebo implant (22 in 2004 and 32 in 2006). On the day of implantation cort- and placebo-nestlings did not differ with respect to age (mean ± SD: 25.9 ± 0.56 days vs. 25.6 ± 0.75 days; Student's t-test, $t = 0.30$, $df = 110$, $p = 0.76$), body mass (292 ± 6.19 g vs. 286 ± 9.68 g; Student's t-tests: $t = 0.56$, $df = 110$, $p = 0.58$), wing-length (111 ± 3.78 mm vs. 108 ± 4.98 mm; Student's t-test: $t = 0.54$, $df = 106$, $p = 0.59$) and sex-ratio (Chi-test, $X^2 = 0.07$; $p = 0.785$). The Swiss committee for animal research

approved the study (animal experiment permit from the 'Service vétérinaire du canton de Vaud' n° 1736).

Plasma corticosterone

To monitor the effect of the implants blood samples of all experimental nestlings were taken before implantation, two, and 20 days after implantation and plasma concentration of corticosterone determined. Blood samples were taken by puncturing the brachial vein and collecting the blood with heparinised capillary tubes. The blood was immediately centrifuged and the plasma stored in liquid nitrogen. After transport to the laboratory, the samples were stored at –20°C until analysis. Since a significant increase of circulating corticosterone levels was observed three minutes after capture of the nestlings (own observation and Romero and Reed, 2005), all blood samples collected within three minutes after capturing the nestlings were considered baseline samples and used in further analysis. Plasma corticosterone concentration was determined using an enzyme immunoassay (Munro and Stabenfeldt, 1984; Munro and Lasley, 1988). Corticosterone was extracted from plasma with 4 ml dichlormethane (5 µl plasma diluted with 195 µl water). All samples were run in triplicates. The dilution of the corticosterone antibody (Chemicon; cross-reactivity: 11-dehydrocorticosterone 0.35%, progesterone 0.004%, 18-OH-DOC 0.01%, cortisol 0.12%, 18-OH-B 0.02% and aldosterone 0.06%) was 1:8'000. HRP (1:400'000) linked to corticosterone served as enzyme label and ABTS as substrate. The concentration of corticosterone in plasma samples was calculated by using a standard curve run in duplicates on each plate. Plasma pools from chicken with a low and a high corticosterone concentration were included as internal controls on each plate. Intra-assay variation ranges from 5 to 13% and inter-assay variation from 12 to 21%, depending on the year and concentration of the internal controls. For details on the assay see Müller et al. 2006.

To estimate free corticosterone, corticosterone-binding globulins (CBG) were measured. CBG capacity was measured using a radioligand-binding assay with tritiated corticosterone (described in Breuner et al., 2003). Briefly, plasma was stripped of endogenous steroids with dextran-coated charcoal solution during 20 minutes. Plasma dilution was optimized for barn owls yielding a dilution of 1:450 and an incubation period of 2 hours and temperature of 4°C. All samples were run in triplicates. Total binding was determined using 50µL buffer (50mM Tris), 50µL ^{3H}CORT (20nM ^{3H}CORT) and 50µL stripped plasma. Non-specific binding was determined using 50µL unlabeled corticosterone (1µM cort) instead of buffer. Glass fiber filters were soaked in 25mM Tris with 0.3% polyethylenimine for 1h before vacuum filtration (Brandel Harvester). Filters were rapidly rinsed with 9ml rinse buffer (25mM Tris; 3 rinses of 3ml). Following filtration

radioactivity bound to the filters was measured by standard liquid scintillation spectroscopy (scintillation cocktail Ultima Gold^{TM} LLT, Perkin Elmer). The equilibrium binding parameters for the specific binding of ^{3H}CORT were determined through equilibrium saturation binding assay of pooled barn owl plasma and ^{3H}CORT concentration between 0.2 and 10.7nM. Affinity estimates (dissociation constant K_d) of corticosterone for CBG in barn owls were 4.11 ± 0.34 nM. Individual hormone binding capacity was estimated using point sample analysis. Percentage CBG bound in the assay was estimated using concentration of ^{3H}CORT and K_d from the saturation analysis with the following formula: % bound = $[^{3H}CORT]/([^{3H}CORT]+K_d)$ and ranged from 77% to 83%. Before estimating free hormone levels, all point samples were corrected to 100% for analysis. Free hormone levels were estimated according to the equation in Barsano and Baumann (1989). A plasma standard was included in all CBG assays which yielded intra-assay coefficients of variation of 6% and an inter-assay coefficient of variation of 23%.

Assessment of plumage traits

Shortly before fledging we recorded plumage traits in all nestlings. Also all breeding adults were captured and plumage traits recorded. We compared the phaeomelanin-based coloration of breast, belly, one flank and one underside of the wing with eight colour chips, ranging from 1 for reddish-brown to 8 for white. To assess the variation in number and size of black spots (eumelanin-based coloration), we placed on the same four body parts a frame of 60 x 40 mm, counted the number of spots and measured its diameter to the nearest 0.1 mm. For each body part we calculated a mean spot diameter, called ‘spot diameter’ and the mean number of spots, called ‘spot number’. For each bird we then calculated a mean coloration, spot diameter and spot number. For details about the method see Roulin (2004). Assessment of plumage traits is reliable (Roulin and Dijkstra, 2003; Roulin, 2004).

The sex of nestlings was determined using the CHD-gene method (for details see Roulin et al., 1999). Breeding females were recognised by the presence of a brood patch.

Statistical procedure

All statistical tests were done using the statistical software package R version 2.4.1 (R Development Core Team, 2006). P-values < 0.05 were considered as significant. Means ± SE are quoted if not otherwise indicated.

Results

Total corticosterone

Baseline total corticosterone levels differed between the implant-groups (cort- vs placebo-nestlings) depending on sampling day, between the implant-groups depending on the spot-diameter of the genetic mother, and between the implant-groups depending on the year as shown by the significant interactions of 'implant × days', 'implant × spot-diameter genetic mother', and 'implant × year' (Table 1; Fig. 1). Spot-diameter of the rearing mother, genetic father, and rearing father, and the nestling itself did not significantly explain variation in baseline corticosterone levels, neither did phaeomelanin-based coloration.

Table 3. Mixed-effect models with total corticosterone, and free corticosterone as dependent variables. As random factors we included nestling identity nested in rearing site. Degree of freedom, F-, and p-values are reported. The analysis of total corticosterone is based on 289 measurements in 120 nestlings of 48 different broods at three different days. The analysis of free corticosterone is based on 270 measurements in 120 nestlings of 48 different broods at three different days.

	total corticosterone			**free corticosterone**		
	df	**F**	**p**	**df**	**F**	**p**
intercept	1,152	626.16	<0.001	1,142	229.69	<0.001
year	2,42	9.85	<0.001	1,42	30.17	<0.001
implant	1,67	43.62	<0.001	1,69	11.23	0.001
day	3,152	34.51	<0.001	3,142	11.72	<0.001
spot-diameter genetic mother	1,42	5.20	0.028	1,42	13.52	<0.001
year*implant	2,67	3.63	0.032			N.S.
year*spot-diameter genetic mother	2,42	5.04	0.011	2,42	7.13	0.002
implant*day	3,152	39.24	<0.001	3,142	13.01	<0.001
implant*spot-diameter genetic mother	1,67	4.90	0.030	1,69	7.38	0.008
day*spot-diameter genetic mother			N.S.	3,142	5.64	0.001

Post hoc tests showed that baseline total corticosterone levels at the day of implantation was not significantly different between cort- and placebo-nestlings (post-hoc mixed-effect-model; 'implant': $F_{1,53} = 1.37$, $p = 0.248$) and did not depend on spot-diameter of the genetic mother (mixed-effect-model; 'spot-diameter genetic mother': $F_{1,34} = 2.66$, $p = 0.112$). Two days after implantation baseline total corticosterone levels of cort-nestlings of small-spotted mothers in 2004 (Fig. 1) were significantly higher than those of cort-nestlings of large-spotted mothers (mixed-effect-model; 'implant × spot-diameter of the genetic mother': $F_{1,39} = 7.02$, $p = 0.012$). In 2006 (Fig. 1) this interaction was not significant (mixed-effect-model; 'implant × spot-diameter genetic mother': $F_{1,26} = 1.80$, $p = 0.192$). 20 days after implantation baseline total corticosterone levels did not differ between cort- and placebo nestlings (mixed-effect-model; 'implant': $F_{1,42} = 0.21$, $p = 0.649$) and did not depend on the spot-diameter of the genetic mother (mixed-effect-model; 'spot-diameter genetic mother': $F_{1,32} = 0.27$, $p = 0.609$).

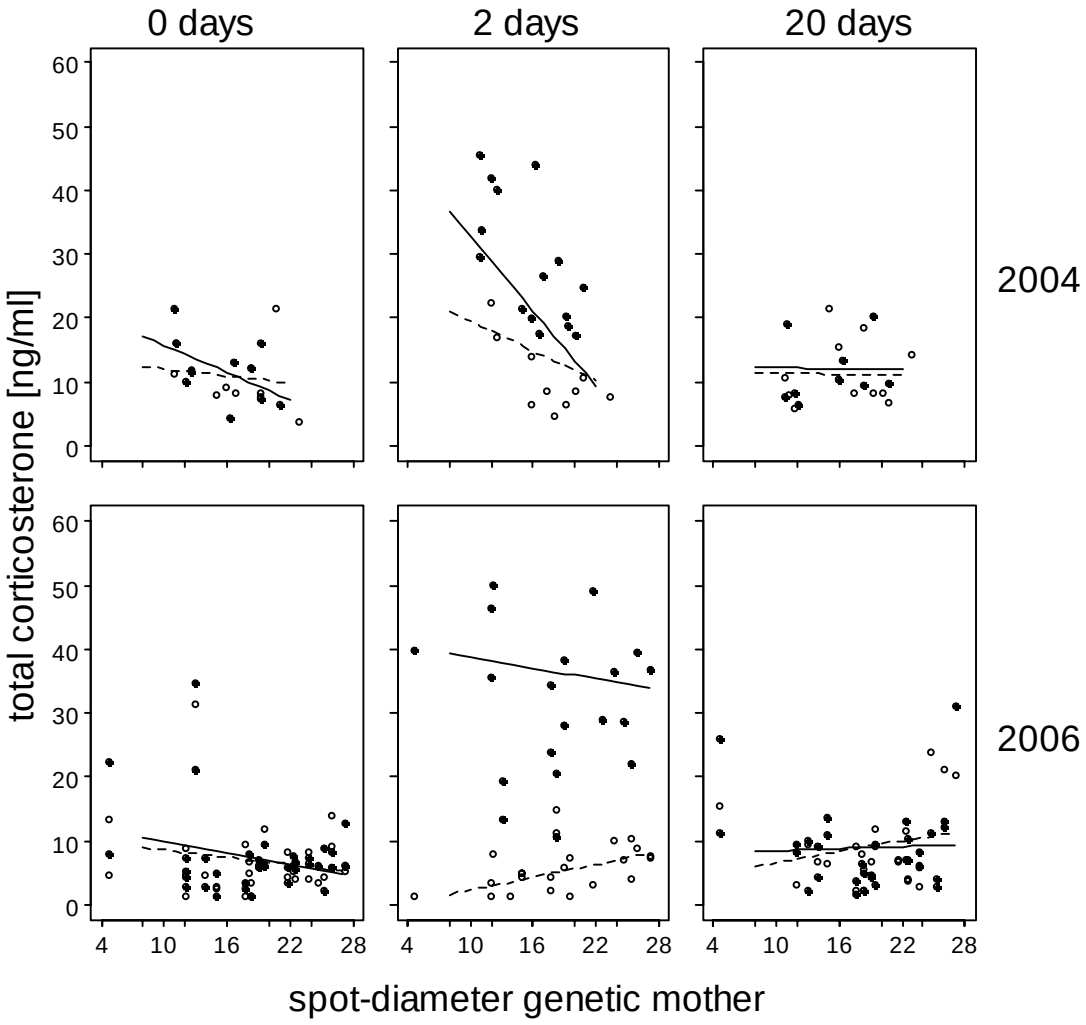

Fig 1. Total corticosterone levels of nestlings in 2004 and 2006 just before implantation (0 days), 2 and 20 days after implantation of cort- (dots) and placebo-nestlings (circles) in relation to spot-diameter of the genetic mother. Lines represent the model-predicted corticosterone levels of cort (line) and placebo-nestlings (dashed line).

Free corticosterone

Baseline free corticosterone levels differed between the implant-groups (cort- vs placebo) depending on the sampling day, and between the implant-groups depending on the spot-diameter of the genetic mother as show by the significant interactions of 'implant × days', and 'implant × spot-diameter genetic mother' (Table 1; Fig. 2). Furthermore, baseline free corticosterone varied between the years depending on the spot-diameter of the genetic mother, and between the days

depending on the spot-diameter of the genetic mother as shown by the significant interactions of 'year × spot-diameter genetic mother', and 'day × spot-diameter genetic mother' (Table 1, Fig. 2). Because the interaction between year and implant was not significant, data of the two years were merged into one graph. Spot-diameter of the rearing mother, genetic father, rearing father, and nestlings did not significantly explain the variation in baseline corticosterone levels, neither did phaeomelanin-based coloration.

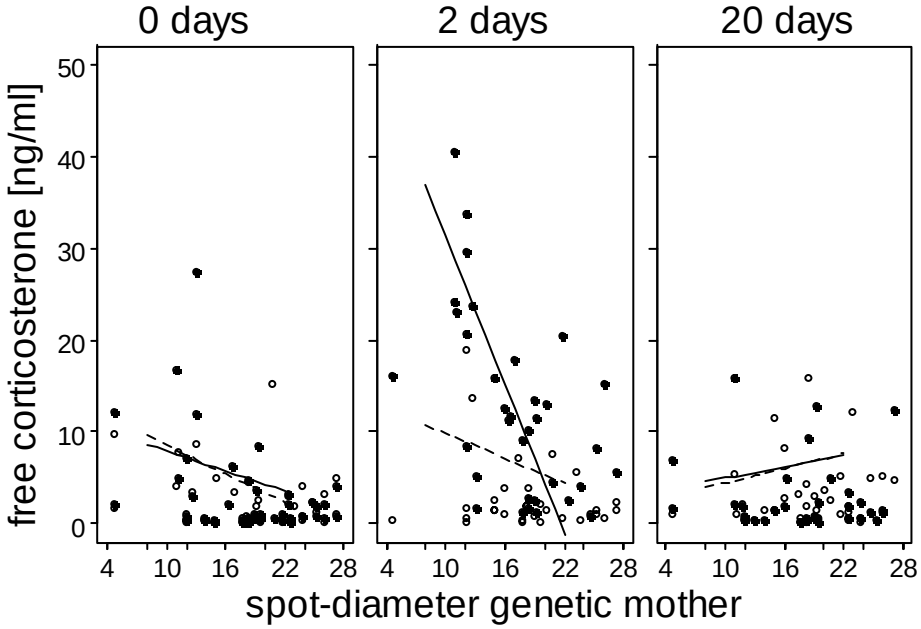

Fig 2. Freel corticosterone levels of nestlings in 2004 and 2006 just before implantation (0 days), 2 and 20 days after implantation of cort- (dots) and placebo-nestlings (circles) in relation to spot-diameter of the genetic mother. Lines represent the model-predicted corticosterone levels of cort (line) and placebo-nestlings (dashed line).

Post-hoc test showed that baseline free corticosterone levels at the day of implantation were not significantly different between cort- and placebo-nestlings (mixed-effect-model; 'implant': $F_{1,51} = 0.64$, $p = 0.429$) and did not depend on the spot-diameter of the genetic mother (mixed-effect-model; 'spot-diameter genetic mother': $F_{1,33} = 3.83$, $p = 0.060$). Two days after implantation baseline free corticosterone levels of cort-nestlings with small-spotted mothers were higher than of cort-nestlings with large-spotted mothers and placebo-nestlings (mixed-effect-model; 'implant × spot-diameter genetic mother': $F_{1,58} = 7.05$, $p = 0.010$). 20 days after implantation baseline free

corticosterone levels did not differ between cort- and placebo nestlings (mixed-effect-model; 'implant': $F_{1,34} = 0.31$, $p = 0.584$) and did not depend on the spot-diameter of the genetic mother (mixed-effect-model; 'spot-diaemter genetic mother': $F_{1,31} = 0.80$, $p = 0.379$).

Discussion

We manipulated corticosterone levels in barn owl nestlings in two different years to examine whether we could explain the individual variation in the increase in circulating corticosterone with phenotypic traits, which are related to fitness-relevant components. Overall we found marked inter-individual variation in the corticosterone increase after artificial administration of corticosterone, which could be partly explained by maternal phenotypic traits. Free corticosterone after artificial corticosterone administration was lower in nestlings from mothers with large spots. Total corticosterone showed the same relationship only in 2004 and not in 2006. 2004 was a good barn owl year with 47 pairs breeding of which 39 raised at least one fledgling successfully and nestlings were in good body condition. In 2006 only 29 pairs started to breed and 21 raised successfully their brood until fledging, body mass of the nestlings was lower than in 2004. Mean total corticosterone increase after artificial corticosterone administration was higher in 2006 (53 ± 7 ng/ml) than in 2004 (29 ± 3 ng/ml). It seems that the differential effect of the corticosterone implants can only be seen in total corticosterone levels when the stress response is not too strong, whereas the free corticosterone level, the fraction which is suggested to be biologically active (Mendel, 1989), always correlated with eumelain-based coloration of the mother.

Differential effect of corticosterone

Nestlings from mothers with large spots had lower corticosterone levels but only under stressful conditions (simulated by corticosterone administration). The corticosterone implants used in this study were designed to release corticosterone constantly over a period of three to seven days. Since an external stressor does not exist and the environmental conditions do not match the animal's experimentally elevated stress hormone level, the animals should try to bring the corticosterone levels down to baseline again. Corticosterone is cleared from circulation by excretion through the bile and the urine, and endogenous production of corticosterone can be down regulated through a negative-feedback mechanism. Variation in the increase of corticosterone levels after artificial administration can therefore either be due to variation in clearance rate or the negative-feedback mechanism of nestlings. Variation in clearance rate or feedback mechanism can be a heritable trait, which, in the barn owl, seems to be signalled in eumelanin-based coloration of the mothers. That such differences in the effects of elevated corticosterone levels can have fitness-relevant consequences has been shown in two recent studies. Less eumelanic barn owl males provision their

brood at a higher rate, but when corticosterone was artificially elevated they reduce their provisioning rate much more than more eumelanic males (Almasi et al., 2008). Furthermore, elevated corticosterone levels reduce nestling growth more in less eumelanic birds than in more eumelanic birds (Almasi et al. in preparation). More eumelanic females breed at a younger age (Roulin and Altwegg, 2007), have nestlings with less ectoparasite and a better humoral immunocompetence (Roulin, 2004) and a more symmetric wing length growth (Roulin et al., 2003). This suggests that the benefit of being heavily spotted is condition dependent and only in stressful conditions the fitness of nestlings from large spotted mothers is better than of nestlings from small spotted mothers. Sexual selection through male mate choice for more eumelanic females (Roulin, 1999) might therefore favour darker females only under more stressful environmental conditions. This could maintain the large variation in the degree of eumelanin-based coloration in European barn owls (Roulin, 2003).

Possible mechanisms

Variation in the sensitivity of nestlings to stress can have different causes. First, mothers with a certain phenotype could pass on genes to the offspring, which makes them better able to cope with stress. Second, mothers with a certain phenotype are in better conditions and produce embryos of better quality. Third, mothers with a certain phenotype could provide better parental care. The third possibility can be ruled out since we cross-fostered all nestlings randomly between mothers with large and small spots. The correlation of nestling corticosterone levels was between nestlings and the genetic mother and not between nestlings and the foster mother.

Possibility one and two cannot be separated with certainty in this study, but we discuss a hypothesis suggested by Ducrest et al. (2008) which predicts that the correlation between the degree of melanism and other phenotypic traits such as stress sensitivity (this study and Almasi et al., 2008), sexual behaviour (West and Packer, 2002), and immune functions (Roulin, 2004) comes from pleiotropic effects of genes regulating melanogenesis. The *POMC*-gene codes for four different melanocortins (α-, β-, γ-MSH and ACTH). α- and β-MSH bind to the Mc1-receptor and trigger eumelanin synthesis (and bind with lower affinity to the four other Mc-receptors which regulate energy homeostasis, immune and cardiovascular functions, sexual behaviour, and stress response). ACTH binds to the Mc2-receptor and stimulates the production of glucocorticoids (but binds also to the Mc1-receptor). The hypothesis that melanocortins mediate covariations between melanin production and other phenotypic traits by binding to Mc-receptors relies on the following assumption: The activity of peptides derived from the *POMC*-gene at the sites where melanin pigments are produced reflects a similar peptide activity in organs where genes coding for the other

phenotypic traits are expressed (Ducrest et al, 2008). The lower free corticosterone levels and the lower total corticosterone levels in the year with good environmental conditions of nestlings with more eumelanic mothers give evidence for a genetic correlation between melanism and stress sensitivity and implies that stress sensitivity is a heritable trait.

We found no direct correlation between nestling eumelanin-based coloration and corticosterone levels. Males are normally less eumelanic than females (Roulin and Dijkstra, 2003) and the correlation between stress sensitivity and eumelanin-based correlation might, therefore, be stronger in females than males. Further is eumealnin-based coloration in the barn owl sex-linked inherited (Roulin and Dijkstra, 2003). In birds females are heterogametic and daughters receive their only Z-gene from their fathers. Mothers resemble, therefore, more their sons than daughters. And genetic imprinting in sons could turn off one copy of the Z-gene. To detect any sex-specific effect on the signalling function of eumelanin-based coloration in nestlings our sample size was most likely too small.

Conclusion

This study provides evidence for a genetic correlation between melanin-based coloration and stress sensitivity. Furthermore, more eumelanic birds are less sensitive to stress, which can be explained by a more sensitive HPA-axis and result in differences in the glucocorticoid response to a stressful situation. Further studies need to investigate directly the variation in the negative feedback mechanisms of more or less eumelanic individuals.

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Chapter 5

Corticosterone mediates the condition-dependent component of melanin-based coloration

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Abstract

The handicap principle of sexual selection theory states that colourful phenotypic traits signal aspects of individual quality because only individuals in prime condition can afford to produce and bear conspicuous traits. Melanin-based pigments participate in the elaboration of many secondary sexual characters and, given their role in sexual selection, melanin-based coloration may, therefore, honestly reflect individual quality. Although the expression of melanism is usually under genetic control, in some species it is condition-dependent. However, the underlying physiological mechanism is yet unknown. Based on the negative feedback link between corticosterone and melanogenesis (melanocortins, tyrosinase) in response to stressful environmental factors, we hypothesize that corticosterone mediates the condition-dependent component of melanism. This hypothesis predicts that stressful factors induce a rise in circulating corticosterone which inhibits the secretion of melanocortins and tyrosinase and in turn melanin production. We tested this prediction by manipulating the level of corticosterone at the time of melanin production in nestling barn owls *Tyto alba*, a species showing heritable variation in the degree of phaeomelanism from reddish-brown to white. The finding that corticosterone-implanted nestlings produced feathers with less phaeomelanic coloration than placebo-nestlings is consistent with the hypothesis that the environment-mediated reduction in the degree of melanism is, at least in part, caused by a rise in corticosterone. In species in which the expression of melanin-based coloration is condition-dependent, we now need a test showing that individuals with less corticosterone and more melanin-based signals are individuals in better condition.

Introduction

Colourful skin, feathers and cuticles are among the most striking visible phenotypic aspects in many animals. Among the different mechanisms responsible for variation in coloration, deposition of melanin pigments is probably the most widespread. Melanin-based coloration is sensitive to both natural and sexual selection by playing an important role in prey-predator interaction, thermoregulation and social interactions (Majerus 1998; Jawor & Breitwisch 2003; Roulin 2004a). Although the expression of melanin-based coloration is often under genetic control (Gantz & Fong 2003; Majerus 1998; Roulin & Dijkstra 2003), a significant environmental component has been detected in several species (e.g. Griffith et al. 1999; Horth 2003; Fargallo et al. 2007) suggesting that the degree of melanism can sometimes honestly signal aspects of individual quality as initially proposed by Zahavi (1975) under the handicap principle of sexual selection. Indeed, in many species of vertebrates melanin-based ornaments are used as criteria in mate choice (Majerus 1998; Jawor & Breitwisch 2003; Roulin 2004a) and the degree of melanism covaries with life history, morphological, behavioural and physiological traits (Roulin 2004a). However, the physiological mechanism that mediates the environmental component of inter-individual variation in melanism is not yet known (Griffith et al. 2006). Identifying the molecules that mediate the environmental component of variation is key because these molecules may affect other important phenotypic traits potentially explaining why less melanic individuals can perform less well than deeply melanic conspecifics (Jensen et al. 2004). To appraise this question, a detailed knowledge of the mechanism underlying the production of melanin pigments is necessary.

Melanin consists principally of two heteropolymers, the brown to black eumelanin and yellow to reddish-brown phaeomelanin that are synthesized in melanocytes in eyes, skin, hair, feathers and cuticle. Melanin synthesis starts with the hydroxylation of L-tyrosine by the rate-limiting enzyme tyrosinase to DOPA and dopaquinone. During eumelanin synthesis the tyrosine-related enzymes TYRP1 and DCT further transform dopaquinone into dihydroxyindole-derived precursors of eumelanin (DHI and DHICA), whereas incorporation of cysteine or glutathione to dopaquinone produces phaeomelanin pigments (Prota 1992). The most important regulators of melanogenesis are the melanocortin 1-receptor (Mc1R), its antagonist the agouti signalling protein (ASIP) and agonists the melanocortins MSHs (melanin-stimulating hormones) and ACTH (adrenocorticotropic hormone) which are posttranslational products of the prohormone encoded by the pro-opiomelanocortin (*POMC*) gene (Eberle 1988). Binding of ASIP and melanocortins to Mc1R triggers the synthesis of phaeomelanin and eumelanin pigments, respectively (Slominski et al. 2004).

Melanocortins regulate not only the production of melanin (Jimbow et al. 2000; Rees 2003), but also a number of other physiological functions including the stress response principally through ACTH, which is part of the hypothalamic-pituitary-adrenal-axis (HPA) (Simpson & Waterman 1988). The HPA consists of the hypothalamic corticotropin-releasing hormone (CRH) that signals to pituitaric ACTH, which further stimulates adrenal glucocorticoid synthesis including cortisol and corticosterone. The latter two glucocorticoids are responsible for a time-limited and adaptive stress response, and they control gluconeogenesis, lipolysis, immune function, sexual activity, growth and development in order to adequately respond to stress (Charmandari et al. 2005; Hofer & East 1998). Glucocorticoids exert their effects through receptors which are expressed in most tissues in vertebrates including the skin and hair follicle cells where melanin pigments are produced and packed (Ito et al. 2005). During the stress response glucocorticoids have inhibiting effects on melanogenesis (Slominski et al. 2004) by reducing the transcription of *POMC*, Mc1R and tyrosinase, and the activity of DCT (Arnold et al. 1975; Ermak & Slominski 1997), and by inducing hair follicle regression (Paus et al. 1994).

Based on the known negative feedback link between corticosterone and melanocortins during the stress response, we tested experimentally in a wild bird whether an elevation in corticosterone level reduces the production of melanin-based coloration. This is an important experiment, because it could reveal that corticosterone mediates the observed environmental effects on melanin production found in other species of birds (house sparrow *Passer domesticus* Griffith et al. 1999; European kestrel *Falco tinnunculus* Fargallo et al. 2007). We therefore manipulated the level of circulating corticosterone in nestling barn owls *Tyto alba* which vary from reddish-brown to white, a phaeomelanin-based trait, and from heavily marked with black spots to immaculate, a eumelanin-based trait (Roulin 2004b). We first demonstrate that inter-individual variation in the degree of reddish-brownness is due to the deposition of phaeomelanin pigments and not of porphyrin and carotenoids, two other reddish pigments. Then, we report an experiment where we implanted subcutaneously pellets of corticosterone or placebo in 15- to 39-day-old nestlings to test the effect of corticosterone on the production of phaeomelanin-based feather coloration. We could not test the effect of corticosterone on eumelanism, because black spots, located at the tip of feathers, were already produced at the time of implantation, while phaeomelanic pigments were still deposited in the growing feathers (feathers are entirely reddish-brown).

Methods

Study organism

In most worldwide distributed barn owl populations, individuals vary in the degree of phaeomelanism from reddish-brown to white and in the degree of eumelanism from heavily marked with black spots to immaculate. Plumage traits are genetically correlated with darker reddish-brown birds displaying more and larger black spots (Roulin & Dijkstra 2003; Roulin 2004b). Nestlings already express the full variation in these two plumage traits, and feathers grown at the nestling stage are not moulted until the second year of age, i.e. after the first breeding attempt. Cross-fostering experiments have demonstrated that the resemblance in coloration between related individuals is heritable and not influenced by the environment at least not to a detectable extent (Roulin& Dijkstra 2003). Although the two sexes can express any plumage trait, females are on average more melanic than males, i.e. darker reddish-brown and displaying more and larger black spots. These two plumage traits appear to play a role in mate choice with males potentially preferring heavily over lightly spotted females (Roulin 1999; Roulin & Altwegg 2007) and females potentially preferring white over reddish-brown males (Roulin & Altwegg 2007). The size of black spots covaries positively with humoral immunocompetence (Roulin et al. 2000), parasite resistance (Roulin et al. 2001a), developmental homeostasis (Roulin et al. 2003), calcium bone concentration (Roulin et al. 2006), age at maturity and survival prospects (Roulin & Altwegg 2007). Darker reddish-brown males were observed to produce more (Roulin et al. 2001b) and heavier offspring (Roulin et al. in press). Therefore, the two melanin-based color traits covary with important physiological and life history traits.

Experimental design

The study was carried out in 2004, 2005 and 2006 in Western Switzerland. To experimentally investigate the effect of corticosterone on the expression of phaeomelanin-based coloration in nestlings, B. Almasi implanted subcutaneously in the flank either a pellet (diameter: 0.5 cm, biodegradable carrier-binder containing 15 mg corticosterone; Innovative Research of America, Florida) releasing corticosterone during four days which resulted in a circulating corticosterone level of about 25 ng/ml (baseline level before implantation was 7.94 ng/ml; B. Almasi unpublished data) or a placebo pellet (biodegradable carrier-binder without corticosterone). A small incision was made and the implant was placed under the skin that was then closed with adhesive tissue (Histoacryl, Braun, Switzerland). In 39 broods we implanted two nestlings with corticosterone (hereafter denoted ‘corticosterone-nestlings’) and two other individuals with placebo (hereafter

'placebo-nestlings'), in two broods we had three corticosterone-nestlings and two placebo-nestlings, in eight broods two corticosterone-nestlings and one placebo-nestling, and in 23 broods 1 corticosterone-nestling and one placebo-nestling. Using molecular methods we determined nestling sex in all but one individual (not enough blood was collected for this individual) (Py et al. 2006).

Because some individuals died or fledged earlier than we anticipated, we collected breast feathers to measure phaeomelanin-based coloration in 108 corticosterone- (58 in 2004, 14 in 2005 and 36 in 2006) and 96 placebo-nestlings (52 in 2004, 13 in 2005 and 31 in 2006) aged 55 days. A similar proportion of female (61 out of 113 experimental females; 54.0%) and male nestlings (47 out of 90 males, 52.2%) were implanted with corticosterone (chi-square test: $X^2_1 = 0.06$, $P = 0.80$). Mean (± SD) age at the time of injection (28 ± 5 days; range = 15-39) did not differ between corticosterone- and placebo-nestlings (mixed model ANOVA with age as the dependent variable and nest of rearing as a random factor, treatment: $F_{1,144.4} = 2.38$, $P = 0.13$). At the time of implantation, corticosterone- and placebo-nestlings did not differ in wing length (mixed model ANCOVA, treatment: $F_{1,147.4} = 2.77$, $P = 0.10$, age: $F_{1,169.7} = 3691.2$, $P < 0.0001$) and body mass (mixed model ANCOVA, treatment: $F_{1,138.9} = 0.25$, $P = 0.62$, age: $F_{1,188} = 42.60$, $P < 0.0001$; age^2 = $F_{1,186.2} = 18.13$, $P < 0.0001$). Finally, parents of corticosterone- and placebo-nestlings did not differ in plumage coloration, number of spots and spot diameter (Student's *t*-tests on mean nest values, all *P*-values > 0.70).

Before implanting pellets, we collected a 20 µl blood sample to measure baseline corticosterone level (ng/ml) by puncturing the brachial vein and collecting the blood with heparinised capillaries. Samples were immediately centrifuged and the plasma stored in liquid nitrogen. After transport to the laboratory, the samples were stored at –20°C until analysis in the next autumn. An increase in circulating corticosterone after an initial stress is detected after three minutes (Romero & Reed 2005; B. Almasi unpublished data). Thus, we measured baseline corticosterone level only if blood samples were collected within three minutes of first opening the nest-box. For this reason, we could measure baseline corticosterone level in 113 of the 204 experimental individuals. Plasma corticosterone concentration was determined using an enzyme immunoassay (Munroe & Stabenfeldt, 1984, Munro & Lasley, 1988) following Müller et al. (2006).

Assessment of plumage coloration, number and size of black spots

A. Roulin measured plumage traits in nestlings and their parents in the field. Plumage coloration was assessed on the breast by comparison with eight colour chips from 1 (dark reddish-brown) to 8 (white) (Roulin 1999); for the human eye, on each feather there is a slight continuous gradation in coloration with the top being darker than the base, and thus coloration was assessed only at the top

of feathers. On the same body part, black spots were also counted within a 60 x 40 mm frame and measured to the nearest 0.1 mm. A mean spot diameter value was calculated and used in the statistical analyses. Assessment of plumage traits was done without being aware of which individual was implanted with a corticosterone or a placebo pellet, since at that time only B. Almasi knew this information.

When nestlings were 55 days of age, we collected three breast feathers of each experimental nestling by cutting fully grown (i.e. metabolically inert) feathers off at their base using a pair of scissors. These feathers were collected at the same place and were representative of all breast feathers, since coloration is uniform on each body part. In November 2006, A. Rossi-Pedruzzi superposed the three feathers and stuck them on a black paper in a black box equipped with a fluorescent tube (8w/20-640 bl-super). A picture of the collected feathers was taken with a digital camera (Konika Minolta, Dimage A200) fixed at a distance of 27 cm from the feathers. Pictures were imported into the software Adobe Photoshop CS2 to measure hue (value that allows a colour to be distinguished as red, blue, yellow, and so on), saturation (or chroma; indicates the purity or strength of a colour) and brightness (indicates the relative lightness of a colour, i.e. the proportion of white and black) on three randomly chosen points at the top and three other points in the middle of the feathers; we took care not to measure coloration of the black spots. For each of these two feather regions, we then calculated a mean value for hue, saturation and brightness. Coloration measured in the field using colour chips was more strongly correlated with saturation (Pearson correlation, $r = -0.69$, $n = 204$, $P < 0.0001$) than with hue ($r = 0.43$, $n = 204$, $P < 0.0001$) and brightness ($r = 0.06$, $n = 204$, $P = 0.40$). To quantify the effect of corticosterone on phaeomelanin-based coloration, we calculated the difference in saturation, hue and brightness between the mean of the three measurements on the top and the mean of the three measurements at the middle of the feathers. This value is denoted ‘contrast in feather saturation’, ‘hue’ and ‘brightness’. We used this procedure because in many cases a decoloured band in the middle of feathers was apparent, probably the effect of corticosterone (see results).

Feather content of melanin, porphyrin and carotenoid

K. Wakamatsu identified the concentration in phaeomelanin and eumelanin pigments in one entire feather collected on the flank of 10 adults. As previously described (Ito & Wakamatsu 1994; Wakamatsu & Ito 2002), melanin pigments were chemically degraded and then using high performance liquid chromatography (HPLC) we quantified pyrrole-2,3,5-tricarboxylic acid (PTCA) which is a specific degradation product of eumelanin, and 4-amino-3-hydroxyphenylalanine (4-AHP) which is a specific degradation product of phaeomelanin. Briefly, 3.0 to 9.0 mg of each

feather were homogenized with 0.5 mL water by using a Ten-Brocke glass homogenizer. For the HPLC determination of eumelanin, feather homogenates (100 µl) were oxidized with potassium permanganate ($KMnO_4$) to give PTCA, which was quantified with HPLC using ultraviolet detection. Each determination was performed in duplicate. For the HPLC determination of phaeomelanin, feather homogenates (100 µl) were hydrolyzed with hydriodic acid (HI) to give 4-AHP which was quantified with electrochemical detection. One nanogram of PTCA corresponds to 50 ng of eumelanin, and 1 ng of 4-AHP to 9 ng of phaeomelanin.

I. Miksík determined the concentration in porphyrins in one feather weighing between 10 and 25 mg collected on the flank or belly of eleven individuals found dead along French highways in 2003. Using a similar method as the one to determine porphyrins in eggshells (Miksík et al. 1996), these feathers were esterified in a solution of 10 ml absolute methanol (LiChrosolv, gradient grade for chromatography, Merck, Darmstadt, Germany) containing 5% concentrated sulphuric acid. This procedure was done at room temperature in the dark under atmosphere of N_2. One day later extracts were decanted and mixed with 2.5 ml chloroform (Merck; chloroform GR, ISO) and 5 ml distilled water before being shaken. The lower chloroform phase was collected and the higher water phase was once again extracted with chloroform. The two extractions were pooled together before being evaporated to dryness and reconstituted in 0.5 ml chloroform. Standard for the quantification (protoporphyrin IX; Sigma, St. Louis, MO, USA) was treated using the same procedure. To extract porphyrins strongly packed in feathers, the remaining feathers were hydrolyzed under alkaline conditions (0.1 M NaOH, 105°C) during 18 hours under N_2, filtered, neutralized with HCl, evaporated and reconstituted in a methanol solution containing 5% concentrated sulphuric acid. This reconstituted solution was then esterified and the extracts collected as described above. In the feather extracts, we determined and quantified protoporphyrin IX in the form of dimethylester. We used a reversed-phase high-performance chromatography using Agilent 1100 LC system (Agilent, Palo Alto, CA, USA) consisting of a degasser, binary pump, autosampler, thermostatted column compartment and multiwavelength and fluorescence detectors. Chromatographic separation was carried out in a Zorbax Eclipse XDB C18 column (150 x 2.1 mm I.D., Agilent). The sample (20 µl) was injected into the column and eluted with a gradient consisting of (A) methanol-water-pyridin 35:65:0.25 v/v and (B) methanol-acetonitrile-pyridine 90:10:0.25 v/v (flow rate 0.25 ml/min, temperature is 55°C). The gradient started at A/B 80:20 reaching 10:90 ratios after 15 minutes. For the next ten minutes the elution was isocratic followed by another ten minutes isocratic elution at 100% B. Elution was monitored by absorbance at 410 nm and by fluorescence at $405_{ex}/620_{em}$ nm.

J.D. Blount analysed barn owl feathers for carotenoid pigments using standard methods (Hudon & Brush 1992; Stradi et al. 1995). Four or five feathers collected from the flank of ten individuals were washed in ethanol (30 sec) and hexane (30 sec), respectively, before being blotted dry on filter paper. Samples (3-5 mg) of the coloured barbules were then cut from feathers and ground to a fine powder using a mixer mill (model MM200 with zirconium oxide jars and balls; Retsch GmbH, Haan, Germany) at 30 Hz for 15 minutes in the presence of 3ml methanol. The resultant mixture was centrifuged at 12,000 g and 4^{o}C for 5 minutes. After centrifugation, the methanol was collected and then evaporated to dryness in a vacuum at room temperature using a sample concentrator. Samples were then redissolved in 1 ml methanol ready for determination of carotenoids if present, which was done using two methods. Firstly, to determine the fine spectrum and wavelength of maximum absorbance (λ_{max}) of samples, the absorbance at 1nm intervals between 400-500 nm was measured using a bench top spectrophotometer (Nicolet Evolution 500; Thermo Electron Corporation, Hemel Hemstead, UK). Secondly, samples (50 µl) were injected into a Dionex HPLC system (Dionex Corporation, California, USA) fitted with a 5 µm ODS guard column and a Spherisorb ODS2, 5 µ C_{18} reverse-phase column (250 x 4.6 mm) (part PSS831915; Waters Corporation, Massachusetts, USA) maintained at 20^{o}C in a thermostatted column compartment (TCC-100; Dionex). The mobile phase consisted of a linear gradient starting with 100% solution A (acetonitrile-methanol, 85:15, v/v) and ending with 100% solution B (acetonitrile-dichloromethane-methanol, 70:20:10, v/v) over 24 minutes at a flow rate of 2 ml minutes^{-1}. Data were collected from 350-600 nm using a Photodiode Array Detector (PDA-100; Dionex). In the case of both absorbance spectrophotometry and HPLC JDB also ran replicates of a positive control (carotenoids extracted from canary feathers in methanol) to validate the isolation and determination procedures.

Statistical analysis

Statistical tests were computed with the JMP statistical package version 6 and *P*-values lower than 0.05 are considered significant. Means are quoted ± 1 SD.

Ethical note

The experiment was under legal authorization of the 'Service vétérinaire du canton de Vaud' (n° 1736). The moderate level of an injection of corticosterone had only a transient effect on nestling body mass (mean ± SE body mass before implantation in 49 corticosterone- and 45 placebo-nestlings in 2004: 331 ± 7 g vs. 323 ± 7 g; Student's *t*-test: $t_{92} = 0.79$, $P = 0.43$; six days later: 322 ± 6 g vs. 344 ± 6 g: $t_{90} = 2.66$, $P = 0.009$), and around fledging (i.e. 50 days of age) there was no

difference in body mass between corticosterone- and placebo-individuals (350.4 ± 3.7 vs. 356.6 ± 3.5 g; Student's *t*-test: $t_{182} = 1.20$, $P = 0.23$). Before fledging a similar proportion of corticosterone- (10 out of 122, 8.2%) and placebo-nestlings (8 out of 114, 7.0%) died (chi-square test: $\chi^2_1 = 0.12$, $P = 0.73$). Because in 2005 we implanted only two nestlings per broods we could test whether implanting a pellet decreased survival between the time of implantation and fledging. This was not the case since 20 out of 179 (11.2%) non-implanted nestlings died while three out of 36 (8.3%) implanted nestlings died ($\chi^2_1 = 0.25$, $P = 0.62$).

Results

Feather content of melanin, porphyrin and carotenoid

Concentration of eumelanin and phaeomelanin in feathers was 486 ± 49.4 and 3751.47 ± 1333.2 ng/mg, respectively. In a stepwise ANCOVA, feather concentration of phaeomelanin was associated with plumage coloration measured on a scale from 1 to 8 ($F_{1,8} = 26.76$, P = 0.0008) but not with number of spots, spot diameter and sex (P-values > 0.47). Feather concentration of eumelanin tended to be higher in females than in males ($F_{1,8} = 4.88$, P = 0.058); plumage coloration, number of spots and spot diameter did not explain any significant variation in eumelanin (P-values > 0.11). Mean feather concentration of porphyrin was 132 ± 68 μg/g. Porphyrin concentration was not significantly correlated with plumage coloration (Pearson correlation: r = -0.20, n = 11, P = 0.55). No carotenoids were detected.

Baseline corticosterone level

Before pellet implantation, mean baseline corticosterone level was 7.94 ± 6.69 ng/ml. This level was not associated with the degree of reddish-brownness (i.e. saturation) measured at the tip of feathers (mixed model ANOVA with log-transformed baseline corticosterone level as the dependent variable and nest as a random variable: $F_{1,108.3} = 1.61$, $P = 0.21$).

Effect of corticosterone on phaeomelanin-based coloration

In 64 out of the 108 (59.3%) corticosterone-nestlings, there was a visible decoloured feather zone located at a distance of 4 to 18 mm (8.7 ± 3.6 mm) from the tip (the collected feather was 20-40 mm long). A similar decoloured zone was visible in only five out of the 96 (5.2%) placebo-nestlings (chi-square test: $X^2_1 = 66.3$, $P < 0.0001$). Decoloured zones were visible on the collected feathers but also on the entire plumage when we had the owls in hand. In corticosterone-nestlings, presence/absence of a decoloured zone was not associated with nestling age at the time of implantation, sex or plumage traits (logistic regression with presence/absence of decoloured zone as

dependent variable (0/1): all *P*-values > 0.51). The distance between the tip of feathers and the decoloured zone was positively correlated with age at implantation ($r = 0.54$, $n = 64$, $P < 0.0001$), which is consistent with the fact that the feather part above the decoloured zone had already been produced at the time of implantation.

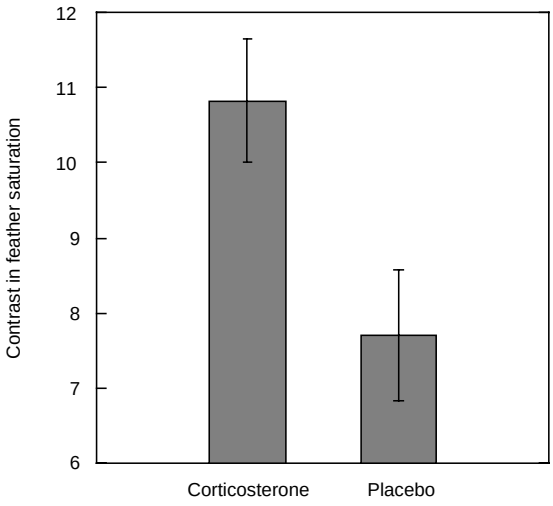

Fig. 1. Contrast in feather saturation between nestlings implanted with corticosterone and placebo. Contrast refers to the difference in saturation between the tip and middle of feathers.

In both placebo- and corticosterone-nestlings, coloration at the tip of feathers was more saturated (i.e. more reddish-brown; 25.0 ± 11.2 vs. 25.11 ± 10.1) than in the middle where the feather was decoloured (17.3 ± 7.5 vs. 14.3 ± 7.0) (paired *t*-tests: placebo-nestlings: $t_{95} = 8.84$, $P < 0.0001$; corticosterone-nestlings: $t_{107} = 13.18$, $P < 0.0001$). Contrast in saturation was more pronounced in female (10.3 ± 8.1) than male nestlings (4.8 ± 8.0; mixed model two-way ANOVA with contrast as the dependent variable and nest as a random variable: $F_{1,198} = 16.51$, $P < 0.0001$) and in corticosterone- than placebo-nestlings (Fig. 1; same model: $F_{1,150.9} = 8.82$, $P = 0.0035$; interaction between sex and treatment was not significant ($P = 0.56$); including year in the model did not modify the results. The effect of our treatment was detected in the middle of feathers where coloration was lighter in corticosterone- than in placebo-nestlings (Student's *t*-test: $t_{202} = 2.97$, $P = 0.0034$), while no treatment effect was detected at the tip of feathers which is consistent with the fact that this feather part had already been produced at the time of implantation ($t_{202} = 0.07$, $P = 0.95$). Within corticosterone-individuals saturation at the tip of feathers was positively correlated with saturation in the middle of feathers (mixed model ANOVA with saturation in the middle of feathers as the dependent variable and nest as a random factor, saturation at the tip of feathers: $F_{1,100.9} = 22.11$, $P < 0.0001$; sex: $F_{1,102.4} = 7.03$, $P = 0.009$, females being darker than males; number of spots: $F_{1,97.82} = 0.20$, $P = 0.66$; spot diameter: $F_{1,102.1} = 1.67$, $P = 0.20$). This indicates that the

reduction in saturation due to corticosterone is proportional to the degree of phaeomelanism in the absence of extra corticosterone but is not proportional to the degree of eumelanism (i.e. number and size of black spots). Corticosterone did not affect feather hue and brightness (results not shown).

Discussion

Phaeomelanism varies gradually from dark reddish-brown at the tip to a lighter coloration at the middle of feathers, a gradient that was more pronounced in corticosterone- than placebo-nestlings. This indicates that corticosterone reduced the deposition of phaeomelanin pigments in the middle of the feathers; because black spots were already produced at the time of implantation (spots are located at the tip of the feathers), we could not test the possibility that this hormone also regulates the deposition of eumelanin pigments. A decoloured feather band was detected in 59.3% of corticosterone-individuals.

Implantation of corticosterone pellets elevated circulating corticosterone to a level of 25 ng/ml on average over a period of four days (baseline level before implantation is 7.94 ng/ml on average), before corticosterone returns to baseline level (B. Almasi unpublished data) indicating that the effect of our experiment should have been transient. Accordingly, the decoloured zone in the middle of feathers of corticosterone-individuals measured approximately 5-10 mm; a similar decoloured zone was observed in only 5.2% of placebo nestlings indicating that in natural conditions decoloration can happen, and thus the production of phaeomelanin pigments can sometimes be condition-dependent in the barn owl. This is consistent with the finding that so-called fault-bars (deterioration of feather structure and pigmentation) on wing and tail feathers have been associated with stressful events (King & Murphy 1984; Murphy et al. 1988, 1989; Machmer et al. 1992).

Based on the genetic mechanisms underlying melanin biosynthesis, we predicted that an injection of corticosterone should decrease the synthesis or deposition of phaeomelanin pigments and in turn reddish-brown feather coloration; this is consistent with the finding that an injection of corticosterone decreased saturation (a measure of the strength of a colour) but neither hue (a value that indicates coloration) nor brightness which indicates the relative lightness of a colour. Melanin pigments are synthesized in lysosome-like organelles called melanosomes (Slominski et al. 2004) which are then transferred from melanocytes into surrounding keratinocytes that are progressively incorporated from the feather germ follicle to the feather filaments along with keratin (Lin 2006). Corticosterone may affect feather pigmentation in two major ways. Firstly, glucocorticoids can inhibit the transcription of tyrosinase, Mc1-R and *POMC* in the feather bud as shown in murine skin (Ermak et al. 1997), and thus synthesis of both phaeomelanin and eumelanin should be reduced in

the feather bud. Accordingly, corticosterone decreased the production of phaeomelanin pigments as shown by the decoloured feather zone; the effect was transient because pellets released corticosterone during only four days, as shown by regular blood sampling (B. Almasi unpublished data). Secondly, because the plasma level of corticosterone was elevated for a period of four days, nestlings were probably in a state of moderate stress. Therefore, the injection of corticosterone may have reduced the level of ACTH through a negative feedback loop. This may have occurred in the pituitary gland, but also in feather buds, because mammalian hair follicle and dermal fibroblast of the skin display a functional HPA-axis equivalent to the hypothalamic-pituitary-adrenal axis (Ito et al. 2005; Slominski et al. 2005). Reduction of *POMC*-derived peptides reduces melanocyte proliferation, migration and principally eumelanin synthesis (Suzuki et al. 1996). This may have led to a decrease in the production or deposition of phaeomelanin pigments. Corticosterone can therefore mediate the condition-dependent component of melanin-based coloration if there is inter-individual variation in corticosterone level or in the sensitivity to the effect of corticosterone, which is the case in several organisms (Koolhaas et al. 1999; Evans et al. 2006). It now remains to investigate the general applicability of our results in other vertebrates and to test whether the effect of corticosterone on the deposition of melanin pigments is stronger in sexually selected than non-sexually selected traits (Cotton et al. 2004).

Although the degree of melanin-based coloration can be under strong genetic control, the feedback loop between the production of ACTH (one of the hormones responsible for melanin production) and corticosterone during the stress response may account for the environmental component of inter-individual variation in the degree of melanism observed in other bird species (Griffith et al. 1999; Fargallo et al. 2007). Interestingly, corticosterone altered phaeomelanin production only when experimentally elevated, while baseline corticosterone level was not correlated with coloration indicating that corticosterone affects melanin production only if elevated during prolonged periods of stress. In the barn owl, although the expression of phaeomelanism is under strong genetic control (Roulin et al. 1998; Roulin & Dijkstra 2003), in 5.2% of the placebo-implanted nestlings we observed a decoloured feather zone. This suggests that a heritable melanin-based trait can sometimes be sensitive to environmental factors.

Although in the barn owl an experimental elevation in corticosterone level resulted in a reduction in the degree of reddish-brownness, a manipulation of brood size did not alter the expression of this trait (Roulin et al. 1998), although manipulating brood size induced a clear change in offspring body condition (Roulin et al. 1999). Furthermore, the degree of phaeomelanism was not associated with the place of nestlings in the within-brood age hierarchy (Roulin & Dijkstra 2003), although late-hatched individuals have more ectoparasites and have a lower access to food

resources than their early-hatched siblings. Because a reduction in food supply can lead to an increase in corticosterone level (e.g. Kitaysky et al. 2001), it appears that in the barn owl a relatively high threshold in corticosterone level has to be reached and during a sufficiently long period of time to result in a significant reduction in melanin-based coloration. Our experimental treatment resulted in a three-fold increase of total corticosterone level during four days, values that are naturally observed after a short starvation period of one night; a stress response to an acute stressor results in a four- to five-fold increase of total corticosterone level (B. Almasi unpublished data). If barn owls appear to be relatively unaffected to stressful factors in their expression of melanin-based coloration, a lower threshold in corticosterone level may be sufficient to induce a change in melanin-based coloration in species such as the house sparrow (Griffiths et al. 1999) and European kestrel (Fargallo et al. 2007) in which environmental conditions can lead to a clear change in the expression of eumelanin-based colour traits. Apparently, directional sexual selection exerted on the degree of eumelanism in these two bird species may increase the susceptibility to corticosterone, a proposition that deserves further testing.

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Chapter 6

Regulation of free corticosterone and CBG capacity under different environmental conditions in altricial nestlings

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Abstract

The concentration of circulating glucocorticoids is regulated in response to environmental and endogenous conditions. Total circulating corticosterone, the main glucocorticoid in birds, consists of a fraction which is bound to corticosterone-binding globulins (CBG) and a free fraction. There is increasing evidence that the environment modulates free corticosterone levels through varying the concentration of CBG, but experimental evidence is lacking. To test the hypothesis that the regulation of chronic stress in response to endogenous and environmental conditions involves variation in both corticosterone release and CBG capacity, we performed an experiment with barn owl (*Tyto alba*) nestlings in two different years with pronounced differences in environmental conditions and in nestlings experimentally fed *ad libitum*. In half of the individuals we implanted a corticosterone-releasing pellet to artificially increase corticosterone levels and in the other half we implanted a placebo pellet. We then repeatedly collected blood samples to measure the change in total and free corticosterone levels as well as CBG capacity. The increase in circulating total corticosterone after artificial corticosterone administration varied with environmental conditions and with the food regime of the nestlings. The highest total corticosterone levels were found in nestlings growing up in poor environmental conditions and the lowest in *ad libitum* fed nestlings. CBG was highest in the year with poor environmental conditions, so that, contrary to total corticosterone, free corticosterone levels were low under poor environmental conditions. When nestlings were fed *ad libitum* total corticosterone, CBG and free corticosterone did not increase when administering corticosterone. These results suggest that depending on the individual history an animal experienced during development the HPA-axis is regulated differently.

Introduction

In order to mediate adaptive physiological and behavioural responses to changes in the animal's life, the concentration of circulating glucocorticoids is regulated in response to environmental and endogenous conditions (Boonstra et al., 1998; Kitaysky et al., 2001a; e.g. Romero, 2002; Cockrem and Silverin, 2002; Rogovin et al., 2003; Kuznetsov et al., 2004). Both baseline and stress-induced glucocorticoid levels can vary diurnally, and seasonally, by environmental condition or body energy stores (Romero and Wingfield, 1998; Romero et al., 2000; Breuner and Orchinik, 2001; Romero, 2002; Love et al., 2004; Romero et al., 2006). Glucocorticoid levels are regulated through negative feedback: elevated glucocorticoids interact with neural receptors which reduce further glucocorticoid secretion of the adrenal (Carsia and Harvey, 2000). If the stressor persists and glucocorticoids remain elevated, the negative feedback ceases to function and chronic elevation of glucocorticoids begins (e.g. Young et al., 1995).

To appraise the role of corticosterone in shaping an adaptive response to stress, the majority of researchers quantify only total levels of circulating corticosterone (corticosterone is the primary glucocorticoid in non-mammalian tetrapods). However, downstream elements (such as plasma binding globulins or cellular receptors) in this pathway may also regulate the physiological and behavioural outcome of a stress response. Recent evidence indicates that plasma corticosteroid binding globulin (CBG) may affect the organismal outcome through regulation of free hormone levels. CBG is a glycoprotein specific primarily for glucocorticoids and progestins (although it also binds androgens with relatively high affinity) (Westphal, 1983). The free hormone hypothesis (Mendel, 1989) posits that plasma corticosterone bound by CBG is unavailable to enter tissues. Therefore, free, unbound hormone would represent the biologically relevant fraction of hormone in the plasma (Westphal, 1983; Mendel, 1989; Rosner, 1990). Corticosterone bound to CBG may have its own function: at sites of inflammation serine proteases are secreted, cleave CBG which releases corticosterone and thereby increases the local concentration of free corticosterone while maintaining low levels of free corticosterone elsewhere (Pemberton et al., 1988). CBG also binds to membrane receptors and activates intracellular-second-messenger systems (Nakhla et al., 1988; Strel'chyonok and Avvakumov, 1991). However, opinions are mixed and there is still very little evidence directly testing whether in different vertebrate classes total, bound, or free hormone concentration is the biologically relevant fraction. Therefore, we believe it is not sufficient to quantify only total corticosterone titers; we indeed have to

distinguish a free fraction, as well as a fraction that is bound to corticosterone-binding globulins (CBG).

There is increasing evidence that the environment modulates free corticosterone levels through varying the concentration of CBG. For example, after 18 hours of food deprivation in white-crowned sparrows (*Zonotrichia leucophrys*) total corticosterone levels are no longer elevated over controls, and a reduced CBG capacity maintains high free corticosterone levels (Lynn et al., 2003). These data suggest that adrenal exhaustion may be solved by subsequent lowering of CBG capacity, maintaining elevated free corticosterone levels without the continued high corticosterone secretion. However, any further increase in corticosterone secretion (e.g. in response to a novel stressor) would then result in an unusually large increase of free corticosterone (Breuner and Orchinik, 2002). A field-based example also comes from white-crowned sparrows: in three separate populations the free corticosterone response measured after 30 minutes of restraint mirrored population differences in number of clutches possible in a season. That is, the population with the highest potential to renest showed the highest free corticosterone level in response to capture and handling; this population might therefore be behaviourally and physiologically more responsive to environmental perturbation (Breuner et al., 2003). An advantage of regulating the availability of circulating free corticosterone through CBG, rather than corticosterone secretion, is that the deleterious effects of a high level of corticosterone can be minimized. However, there might be a cost associated with increasing CBG-capacity. While increased CBG capacity lowers free corticosterone levels it can also bind a larger proportion of other hormones, such as progestins and androgens, which was initially not intended.

Poor nutritional conditions and low body energy reserves generally lead to a glucocorticoid response (e.g. Romero and Wikelski, 2001; Kitaysky et al., 2001a; Jenni-Eiermann et al., 2008). In altricial nestlings, which depend on food brought by their parents, an increase in total circulating corticosterone under nutritional stress may serve to increase begging, competitiveness against siblings and food intake (Kitaysky et al., 2001b). However, elevated total corticosterone levels reduce growth rate (Baron et al., 1994; Love et al., 2003; Hull et al., 2007) and immunity (Bourgeon and Raclot, 2006). Therefore, in order not to compromise growth, nestlings should avoid high total corticosterone levels and may regulate the availability of free corticosterone by varying the capacity of CBG. To our knowledge there is no study that measured total and free corticosterone as well as CBG capacity in free-living birds under various levels of stress. Here, we report such a study in nestling barn owls

(*Tyto alba*) to test the hypothesis that poor rearing condition during growth influences the ability of nestlings to cope with a novel stress situation. We, therefore, repeatedly measured total and free corticosterone levels as well as CBG capacity in individuals raised under different feeding conditions and for which we experimentally manipulated the level of circulating corticosterone. To this end we considered nestlings from two years with good and poor food conditions which resulted in a pronounced difference in nestling body condition. In a group of nestlings we provided food *ad libitum*. The latter treatment was useful to disentangle the effect of elevated corticosterone itself from a possibly higher food demand or lower food intake of nestlings as a response to an experimental elevation of corticosterone levels. To increase total corticosterone concentrations to levels comparable to those reached by a strong stressor, we implanted individuals with corticosterone-releasing pellets; control nestlings were implanted with placebo pellets. We predict that when nestlings are well fed and hence rearing conditions are relaxed, nestlings should be better able to cope with an experimental elevation of corticosterone, i.e. have a better regulation of endogenous corticosterone production and clearance rate and are, therefore, able to maintain total corticosterone level in a normal range. In contrast, when individuals are food-restricted they may not be able to lower total corticosterone to normal levels and CBG capacity may increase to keep free corticosterone in a normal range.

Methods

Study animal

The barn owl is a medium-sized predator of small mammals (99% of the diet; Roulin, 2004). Two to eleven eggs are laid between February and August, and eggs hatch asynchronously on average every second to third day creating a pronounced within-brood age hierarchy. The abundance of the main food, wood mice and voles, determines breeding density, clutch size and the proportion of birds producing two annual clutches (personal observation). Mice and vole abundance, among others, depends on precipitation. When fields are flooded, population size of mice and voles decreases dramatically. Maximal growth rate of the nestlings starts when nestlings are around 17 days old and they lose weight from 40 days of age until fledging at *ca.* 56 days. Body mass recession is spontaneous and nestlings fed *ad libitum* or under restricted food supply can fledge with a similar body mass (Durant and Handrich, 1998). Three-week-old nestlings are thermo-independent and hence no longer brooded by their

mother, which roosts at some distance from the nest at daytime. Nestlings return to the nest until they reach 11-14 weeks of age before dispersing.

Study area and experimental design

The study was carried out in 2004 and 2006 in an area of 190 km^2 in Western Switzerland (46°49'N, 06°56'E) where 110 nest-boxes are available for breeding barn owls. Data on precipitation were obtained from the weather station in Payerne (46°48'N, 06°56'E) in the study area.

We selected the four oldest nestlings in 28 first annual broods in 2004 (hereafter 2004 cohort) and in 21 first annual broods in 2006 (hereafter 2006 cohort). Two of them, randomly chosen, were implanted subcutaneously with a corticosterone pellet (hereafter cort-nestlings) and the two other nestmates were implanted with a placebo pellet (hereafter placebo-nestlings). Individuals from five two-chick broods and from eight three-chick broods were randomly allocated to the cort- and placebo-treatments. The pellets (diameter 5 mm) are made up of a biodegradable carrier-binder containing 15 mg corticosterone or, for placebo, only of the biodegradable carrier-binder (Innovative Research of America, Sarasota, Florida). We implanted the pellet under the skin of the flank above the knee through a small incision, which was closed with tissue adhesive (Histoacryl, Braun, Germany). Since alcohol strongly accelerates the release of corticosterone of the pellets, we never used alcoholic disinfectants. The implants were specified for rats with a given constant release rate of 7 days. At the time of implantation mean ± SE age of cort-nestlings was 29 days ± 0.47 in 2004 (n = 49) and 24 days ± 0.53 in 2006 (n = 41), and of placebo-nestlings 29 days ± 0.74 in 2004 (n = 45) and 23 days ± 0.61 in 2006 (n = 37); these ages were not significantly different between cort-nestlings and placebo-nestlings (mixed-effect-model: $F_{2,214} = 0.343$, $p = 0.71$). Wing length was not significantly different between the two years, cort-, and placebo-nestlings (mixed-effect-model: $F_{2,214} = 0.51$, $p = 0.59$) or sex-ratio (Chi-test, all $\chi^2 > 0.3$).

In 2004 we supplied six second annual broods with food *ad libitum* (hereafter *ad libitum* cohort). Again two of the four oldest nestlings were implanted with a corticosterone implant and the two others with a placebo implant. During the first six days after implantation we put 11 ± 0.5 laboratory rats in the nest-boxes so that in the next morning there were still 3.5 ± 0.5 rats. Mean age of the twelve cort-nestlings and twelve placebo-nestlings was 32 ± 1.14 days and 31 ± 1.81 days, respectively (Student's t-test, $t_{17} = 0.72$, $p = 0.48$); at this age nestlings are able to consume small mammals by themselves. Wing length (t-test, $t_{16} =$

0.7166, p > 0.39), body mass (t-test, t_{17} = 0.7166, p > 0.48), and sex ratio (Chi-test, χ^2 > 0.71) did not differ between the treatments.

Blood samples were taken just before implantation (day 0), two and 20 days after implantation. As soon as possible after opening the nest-box, a blood sample of all nestlings was taken by puncturing the brachial vein and collecting the blood with heparinised capillary tubes. The blood was immediately centrifuged and the plasma stored in liquid nitrogen. After transport to the laboratory, the samples were stored at –20°C until analysis. All methods described in this study were approved by the Swiss committee for animal research ('Service vétérinaire du canton de Vaud' n° 1736).

Total corticosterone assay

Plasma total corticosterone concentration was determined using an enzyme-immunoassay (Munro and Stabenfeldt, 1984; Munro and Lasley, 1988) following Müller et al. (2006). 5 µl plasma was added to 195 µl water, and from this solution we extracted corticosterone with 4 ml dichlormethane, which was re-dissolved in phosphate buffer and measured in triplicate in the enzyme-immunoassay. The dilution of the corticosterone antibody (Chemicon; cross-reactivity: 11-dehydrocorticosterone 0.35%, progesterone 0.004%, 18-OH-DOC 0.01%, cortisol 0.12%, 18-OH-B 0.02% and aldosterone 0.06%) was 1:8'000. We used HRP (1:400'000) linked to corticosterone as enzyme label and ABTS as substrate. The concentration of corticosterone in plasma samples was calculated by using a standard curve run in duplicate on each plate. Plasma pools from chicken with a low and a high corticosterone concentration were included as internal controls on each plate. Intra-assay variation ranges from 5 to 13% and inter-assay variation from 12 to 21%, depending on the concentration of the internal control.

Corticosterone-binding globulin assay

Plasma corticosterone binding globulin (CBG) capacity were measured using a radioligand-binding assay with tritiated corticosterone (described in Breuner et al., 2003). Briefly, plasma was incubated with dextran-coated charcoal solution (20 minutes at room temperature) to strip endogenous steroids. Plasma dilution was optimized for barn owls yielding a dilution of 1:450 and an incubation period of two hours. Outside the stripping process plasma and all assay buffers were maintained at 4°C. All samples were run in triplicates. Total binding was determined using 50µL buffer (50mM Tris), 50µL ^{3H}CORT (20nM ^{3H}CORT) and 50µL stripped plasma. Non-specific binding was determined using 50µL unlabeled corticosterone

(1µM cort) instead of buffer. Glass fiber filters were soaked in 25mM Tris with 0.3% polyethylenimine for one hour before vacuum filtration (Brandel Harvester). Filters were rapidly rinsed with 9ml rinse buffer (25mM Tris; 3 rinses of 3ml). Following filtration radioactivity bound to the filters was measured by standard liquid scintillation spectroscopy (scintillation cocktail Ultima Gold^{TM} LLT, Perkin Elmer). The equilibrium binding parameters for the specific binding of ^{3H}CORT were determined through equilibrium saturation binding assay of pooled barn owl plasma and ^{3H}CORT concentration between 0.2 and 10.7nM. Affinity estimates (dissociation constant K_d) of corticosterone for CBG in barn owls were 4.11 ± 0.34 nM (Fig.1). Individual hormone binding capacity was estimated using point sample analysis, that is, measuring CBG capacity using one concentration of 3H-CORT. Percentage CBG bound in the assay was estimated using the following formula: % bound = [^{3H}CORT]/([^{3H}CORT]+K_d). Levels ranged from 77% to 83%; all point samples were corrected to 100% for analysis. A plasma standard was included in all CBG assays which yielded intra-assay coefficients of variation of 6% and an inter-assay coefficient of variation of 23%.

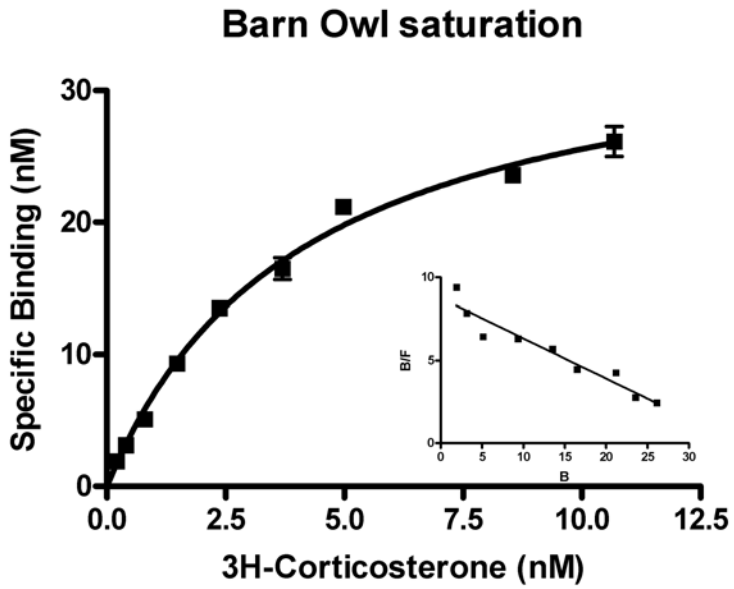

Fig 3. Equilibrium saturation binding curve demonstrating specific binding of ^{3H}CORT to barn owl plasma as a function of increasing concentration of ^{3H}CORT. Points represent means ± SE. Inset: Scatchard-Rosenthal replot of the data, with B = bound- and F = free-^{3H}CORT fraction.

Free corticosterone level

Free hormone levels were calculated according to the equation from Barsano and Baumann (1989):

$$H_{free} = 0.5 \times (H_{total} - B_{\max} - 1/Ka \pm \sqrt{(B_{\max} - H_{total} + 1/K_a)^2 + 4(H_{total}/K_a)})$$

where H_{free} is free hormone, H_{total} is total hormone, B_{max} is total CBG capacity (100% binding), and K_a = 1/ dissociation constant (K_d) (all values in nM).

Statistics

To test the effect of food supply and implantation of a corticosterone pellet on the plasma concentration of total corticosterone, free corticosterone and CBG capacity, we performed for each of these three dependent variables a separate mixed effect ANOVA type III analysis with sex, cohort (2004, *ad libitum*, 2006), days (day 0, 2 and 20 post-implantation) and treatment (corticosterone vs. placebo) as categorical variables and nestling identity nested in nest as random factor to control for pseudo-replication. We included all possible 2- and 3-way interactions and final models only contained significant effects, and main effects involved in significant interactions. All dependent variables were ln-transformed to obtain normality of errors. Within three minutes of first opening the nest-box, total corticosterone levels of nestlings did not increase significantly with time and therefore represent baseline levels of corticosterone (Pearson's correlation between time after opening nest-boxes and total corticosterone level measured before implantation: r = 0.073, df = 166, p = 0.345). For all analyses of total and free corticosterone we thus used blood samples taken below three minutes after first opening the nest-box. CBG capacity did not increase or decrease within the first 15 minutes after first opening the nest-box (Pearson's correlation: n = 901, r = -0.028, p = 0.400), neither does it change with nestling age (Pearson's correlation: age range is 14 to 62 days, n = 900, r = -0.026, p = 0.437). P-values < 0.05 were considered significant for the repeated mixed-effect-models. For post-hoc tests the significance level of p was adjusted to 0.008 after Bonferroni (Sokal and Rohlf, 1995). Means are quoted ± SE. All statistical tests were done using the statistical software package R version 2.4.1 (R Development Core Team, 2006).

Results

Environmental conditions, population density and body condition of nestlings

Precipitation was lower in spring 2004 (1.9 mm d^{-1} ± 0.5) than in spring 2006 (3.2 mm d^{-1} ± 0.8). Number of breeding pairs varied largely between the years. In 2004, 47 pairs were breeding, of which 39 produced at least one fledgling. In 2006, only 29 pairs started to breed and 21 raised their brood successfully (the poorest year in 20 years of study, no second broods). In summer 2004, during the period of second broods, precipitation was high (2.5 mm d^{-1} ± 0.6).

Just before implanting a corticosterone- or placebo-pellet, body condition of nestlings varied strongly between the three cohorts (Fig. 2). Nestlings from the 2004 cohort had the highest body condition, followed by nestlings of the 2006 cohort, and then nestlings of the *ad libitum* cohort (body condition of nestlings was measured as residuals of the regression of body mass before treatment on nestling age: 2004 5.63 g ± 3.23, n= 78; 2006 0.19 g ± 3.87, n= 76; 2004 *ad libitum* –25.23 g ± 10.16, n= 18) (ANOVA: $F_{2,118}$ = 5.03, p = 0.008). Nestlings from the 2004-cohort were on average 31 g heavier for their age than the 2004 *ad libitum* cohort nestlings, a difference which is about 10% of mean nestling body mass at that age.

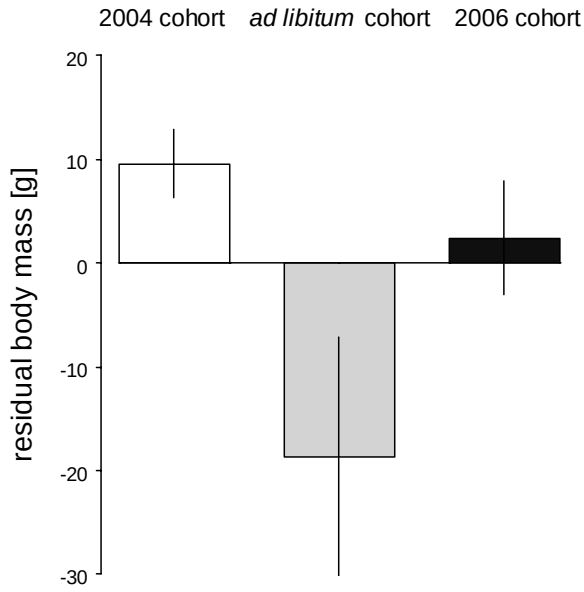

Fig.2. Mean age-corrected body mass of nestlings of the 2004 cohort (white bar), *ad libitum* cohort (grey bar) and 2006 cohort (black bar) before treatment at day 0. Presented are mean residuals ± SE of the relationship between body mass and nestling age. The residuals are calculated with the body mass of 174 nestlings (78 of the 2004 cohort 18 of the *ad libitum* cohort, 76 of the 2006 cohort).

Total corticosterone

Total corticosterone increased after corticosterone implantation but to a different level depending on the cohort. This is shown by the significant interaction of 'cohort by implantation by day' in Table 1A (Fig. 3). Sex had a weak but significant effect on total corticosterone levels but not in interaction with treatment (mean corticosterone of male placebo-nestlings: 8 ng/ml ± 0.7, female placebo-nestling 9 ng/ml ± 0.9).

Total corticosterone levels before implantation (day 0) were similar between the cohorts (post hoc mixed-effect-model: $F_{2,44} = 2.9$, $p = 0.035$ evaluated against a Bonferroni-corrected post-hoc test p-level of $p = 0.008$) and did not differ between nestlings that were later allocated to the cort- and placebo-treatments (mixed-effect-model: $F_{1,60} = 0.8$, $p = 0.363$, Fig. 3). Two days after implantation total corticosterone increased in cort-nestlings in the 2004 (29 ng/ml ± 3) and 2006 cohorts (53 ng/ml ± 7) with cort-nestlings of the 2006 cohort having higher total corticosterone levels than cort-nestlings of the 2004 cohort (mixed-effect-model: $F_{1,7} = 8.1$, $p = 0.007$, Fig. 3). Two days after implantation total corticosterone levels of cort-nestlings of the *ad libitum* cohort (18 ng/ml ± 4) were not increased compared to corticosterone levels before implantation (mixed-effect-model: $F_{1,33} = 5.2$, $p = 0.106$) and there was also no increase in placebo-nestlings from the three cohorts (mixed-effect-model: $F_{1,47} = 0.1$, $p = 0.767$). Twenty days after implantation baseline concentration of total corticosterone of cort-nestlings was back to the level of placebo-nestlings and in the same range as before implantation (mixed-effect-model: $F_{1,57} = 3.5$, $p = 0.067$, Fig. 3).

Corticosterone binding globulin (CBG)

CBG capacity increased two days after implantation only in cort-nestlings of the 2006 cohort and 20 days after implantation CBG capacity was not increased anymore (significant effect of 'cohort' and interaction 'treatment by days' in Table 1 B; Fig 3). Sex had no significant effect on CBG capacity and was thus removed from the final model.

CBG capacity before implantation (day 0) was not significantly different in nestlings that were later allocated to the cort- and placebo-treatments (mixed-effect-model: $F_{1,109} = 4.1$, $p = 0.051$), but significantly higher in nestlings of the 2006 cohort than of the 2004 cohort (mixed-effect-model: $F_{1,46} = 16.5$, $p < 0.001$; Fig 3). There was a large variation in individual CBG capacity in nestlings of the *ad libitum* cohort, but overall the CBG capacity was between the capacity of the 2004 and 2006 cohorts. Two days after implantation there was a significant increase in CBG capacity in the cort-nestlings of the 2006 cohort (mixed-effect-model: $F_{1,39} =$

25.3, $p < 0.001$) but not in cort-nestling of the 2004 and *ad libitum* cohorts after Bonferroni correction (mixed-effect-model: $F_{1,67} = 6.7$, $p = 0.012$). Twenty days after implantation there was no difference in CBG capacity between cort- and placebo nestlings of the 2006 cohort (mixed-effect-model: $F_{1,41} = 0.7$, $p = 0.403$), and also no difference in CBG capacity before and 20 days after implantation (mixed-effect-model: $F_{1,60} = 5.1$, $p = 0.028$). The difference in CBG capacity between the 2004 and 2006 cohort was still significant 20 days after implantation (mixed-effect model: $F_{1,44} = 26.46$, $p < 0.001$).

Table 4. Results of a mixed-effect model ANOVA with A. total corticosterone (n = 287 observations of 151 individuals from 54 broods), B. CBG capacity (n = 482 observations of 198 individuals from 54 broods) and C. free corticosterone (n = 270 observations of 147 individuals from 55 broods) as dependent variable, sex, the 3 cohorts, treatment (cort- and placebo-implants) and days of the measurement (0, 2, 20 after implantation) as fixed effects. Nestling identity nested in site was introduced as random factor. Only the significant interactions and main factors were included into the final model.

	Independent variables	df	F	p
A. ln(totCort)	intercept	1,124	285.78	<0.001
	sex	1,93	4.57	0.035
	cohort	2,51	2.74	0.074
	implant	1,93	0.15	0.695
	days	2,124	18.03	<0.001
	cohort*implant	2,93	0.39	0.677
	cohort*day	4,124	5.23	<0.001
	implant*day	2,124	9.95	<0.001
	cohort*implant*day	4,124	3.36	0.012
B. ln(CBG)	intercept	1,280	1289.93	<0.001
	cohort	2,51	26.39	<0.001
	implant	1,143	3.05	0.083
	day	2,280	15.55	<0.001
	implant*day	2,280	6.93	0.001
C. ln(freeCort)	intercept	1,115	92.69	<.0001
	cohort	2,51	7.92	0.001
	implant	1,90	0.88	0.351
	day	2,115	10.10	0.0001
	cohort*implant	2,90	3.63	0.031
	cohort*day	4,115	3.82	0.006
	implant*day	2,115	19.56	<.0001

Free corticosterone

Free corticosterone levels increased after corticosterone implantation but only in cort-nestlings of the 2004 and 2006 cohort (significant effect of the interactions of 'cohort by treatment' and 'cohort by days'; Table 1 C, Fig. 3). Sex had no significant effect on free corticosterone concentration and was thus removed from the final model.

Baseline levels of free corticosterone were similar in cort- and placebo-nestlings before implantation (mixed-effect-model: $F_{1,57} = 0.7$, $p = 0.416$), but lower in the 2006 than 2004 cohorts (mixed-effect-model: $F_{1,38} = 16.0$, $p < 0.001$; Fig. 3). Two days after implantation free corticosterone increased in cort-nestlings of the 2004 and 2006 cohorts (mixed-effect-model: $F_{1,36} = 68.5$, $p < 0.0001$), but not in cort-nestlings of the *ad libitum* cohort (mixed-effect-model: $F_{1,5} = 0.3$, $p = 0.866$). Contrary to total corticosterone, the increase in free corticosterone two days after implantation in the cort-nestlings of the 2004 and 2006 cohorts was not significantly different from each other (2004: 14 ng/ml ± 2; 2006: 17 ng/ml ± 3; mixed-effect-model: $F_{1,26} = 2.2$, $p = 0.147$). Twenty days after implantation the three cohorts reached the same level of free corticosterone as before implantation (mixed-effect-model: $F_{1,53} = 0.5$, $p = 0.468$; Fig. 3), with nestlings of the 2006 cohort still having significantly lower free corticosterone levels than the 2004 cohort (mixed-effect model: $F_{1,41} = 16.67$, $p < 0.001$).

Discussion

This study demonstrated in nestlings of an altricial avian species that the regulation of the concentration of circulating total and free corticosterone varies with early postnatal environmental condition or the current food condition. Environmental conditions in 2006 were very poor, as pointed out by the lowest number of breeding pairs in 20 years of studying this barn owl population, no second broods and low nestling body condition before the start of the experiment. Precipitation was frequent in 2006 and many fields flooded so that vole populations were low during the whole breeding season. In 2004 environmental conditions were very favourable for barn owls in spring so that nestling body condition was high. However, in summer precipitation was high, and second broods were raised in a period of low food availability and, consequently, a low body condition.

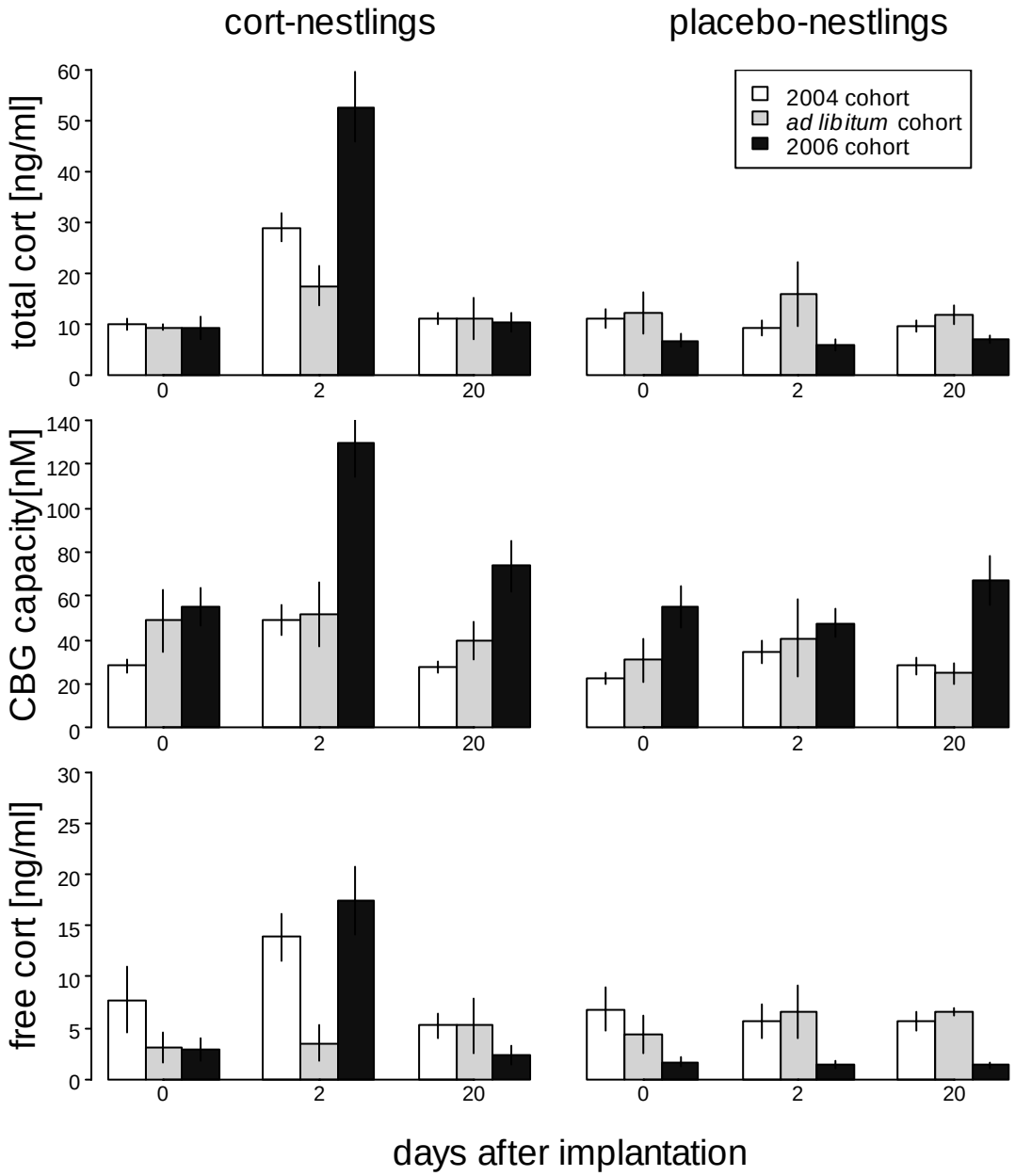

Fig 3. Mean total corticosterone concentration, mean CBG capacity, and mean free corticosterone concentration per cohort ± SE before, 2 and 20 days after implantation. White bars represent 2004, grey bars 2004 *ad libitum*, and black bars 2006 cohort nestlings.

This study suggests two independent mechanisms for regulating available corticosterone levels through (a) variation in endogenous production or clearance rate and (b) variation in CBG capacity. (a) Total corticosterone increase after corticosterone administration varied with environmental conditions. This suggests a change in endogenous production or clearance rates to regulate total corticosterone levels. Hormone implants significantly elevated plasma total corticosterone during a poor condition year, while no effect

of implant was measured during good years. (b) CBG capacity increased, and free corticosterone dropped, when environmental conditions were poor, suggesting that a change in CBG capacity regulated free corticosterone, such as the high CBG capacity leading to lower free corticosterone levels when environmental conditions were poor (2006 cohort), compared with the benign year 2004. CBG capacity increased, and free corticosterone dropped, during the poor 2006 year without corticosterone administration and more dramatically after corticosterone administration.

Regulation of total corticosterone

Total corticosterone levels are often negatively correlated with body condition (Schoech et al., 1997; Kitaysky et al., 1999; Kitaysky et al., 2001a; Pereyra and Wingfield, 2003; Cockrem et al., 2006; Jenni-Eiermann et al., 2008). In this study, nestling body condition before treatment differed markedly between the years with nestlings of the 2004 cohort having a better body condition than nestlings of the 2006 and *ad libitum* cohorts. However, before treatment total corticosterone levels did not differ between the cohorts and, when analysed at an individual level, was not correlated with body condition ($p > 0.6$ for all nestlings together or for each cohort). The lack of a correlation between body condition and baseline total corticosterone level is not surprising. The relationship between body condition and total corticosterone levels is in many cases only seen in capture-induced corticosterone levels (e.g. Smith et al., 1994; Wingfield et al., 1994a; Wingfield et al., 1994b; Schwabl, 1995; Kitaysky et al., 1999) and not in baseline levels.

In contrast to total corticosterone levels before treatment, there was a marked difference between cohorts in the increase of plasma corticosterone levels as a response to the artificial administration of corticosterone. The increase in total plasma corticosterone levels in nestlings exposed to a release of corticosterone from implants over two to four days was lower (i) when nestlings were in good body condition (2004 cohort) and (ii) when they were in poor body condition but given *ad libitum* food (i.e. when any current food stress was eased) and higher when they were in suboptimal body condition (2006 cohort). An artificial administration of corticosterone permits to manipulate the mediator of a stress response without a stressor. Since there is no external stimulus, animals should try to down regulate corticosterone to unstressed levels either through down regulation of endogenous production of corticosterone or increased clearance rate (with this study we were unable to distinguish between the two). The efficacy to down regulate total corticosterone levels may depend on environmental stimuli, which the individual has perceived and currently perceives. Under

relaxed conditions baseline total corticosterone levels (and CBG capacity) are low and individuals should try to counteract an artificial increase of total corticosterone. However, if conditions are suboptimal but not life threatening (e.g. nestlings having previously experienced food shortage) an increase in stress hormone levels might not be compensated in the same way as it would be under relaxed conditions.

Various studies showed that the development of the hypothalamic-pituitary-adrenal (HPA) response to stressful stimuli is altered by early environmental events (reviewed in Caldji et al., 2001) and that the negative-feedback sensitivity can be reduced as a result of early postnatal stress (Navarrete et al., 2007). The large increase in total circulating corticosterone levels after corticosterone administration in the 2006 cohort might be due to a dampened negative feedback mechanism as a result of poor postnatal conditions. Prenatally undernourished rat pups showed a decreased negative feedback after injection of the synthetic glucocorticoid dexamethasone compared to the control group (Navarrete et al., 2007). We, therefore, suggest that the ability to regulate total corticosterone, e.g. clearance rate and negative-feedback mechanism, depends on the current body condition of nestlings but also on the current and past environmental conditions.

Regulation of free corticosterone

Free corticosterone was modulated by changing CBG capacity. This happened in placebo- and in cort-nestlings when environmental conditions were poor. When early and current postnatal environmental conditions were poor (as in nestlings of the 2006 cohort), CBG capacity was higher compared to CBG capacity of nestlings growing up in benign conditions (as in nestlings of the 2004 cohort). With both cohorts having similar total corticosterone levels, this resulted in lower free corticosterone levels in the 2006 than in the 2004 cohort. CBG capacity increased strikingly as a result of corticosterone administration in the year with poor postnatal environmental condition (2006 cohort), but not in the year with the more relaxed environmental condition (2004 cohort). The *ad libitum* food cohort, the cohort with the lowest body condition, showed no increase in total corticosterone after corticosterone administration and hence also no increase in CBG capacity. By the increase in CBG capacity two days after corticosterone implantation, cort-nestlings of the 2006 cohort were able to attain similar free corticosterone levels as cort-nestlings of the 2004 cohort, despite their very high total corticosterone levels.

There is further evidence from the literature that CBG capacity varies with environmental conditions. In tree lizard (*Urosaurus ornatus*) males CBG capacity differed between two years, but no information on environmental conditions or body condition of the animals was given (Jennings et al., 2000). House sparrows (*Passer domesticus*) living in a harsh environment have a higher CBG capacity than birds living in a more benign environment in spring (Romero et al., 2006; but see Romero et al. 1998). Among three populations of white-crowned sparrows, the population with the shortest breeding period and presumably more stressed environmental conditions compared with two other populations experiencing longer breeding seasons has the highest CBG capacity while total corticosterone was the same between the three populations. An additional acute stressor resulted in different free corticosterone concentrations between the populations (Breuner et al., 2003). However, these differences in CBG capacity between populations may have evolved and do not necessarily reflect a plastic response to different environmental conditions.

It thus seems that the role of CBG varies with environmental conditions. Under more risky conditions CBG may act as a buffer to avoid high free corticosterone levels as a result of repeated environmental perturbations. At the same time more corticosterone bound to CBG is available to be directed through CBG-receptors to specific sites. The increase of CBG-capacity after corticosterone administration varies also with environmental conditions. Corticosterone administration induced an increase in CBG capacity only in poor environmental conditions.

Conclusion

The HPA-axis is not a static system, but allows plasticity in its responsiveness. The results of this study confirm that depending on the individual history an animal experienced during development the HPA-axis is regulated differently. Therefore it is important to measure not only total corticosterone but also free corticosterone to understand the different mechanisms that regulate the stress response.

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Chapter 7

Differential suppressive effects of corticosterone on immune components in barn owl nestlings

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Abstract

The energetic demands to cope with stressful situations are traded-off against other physiological functions such as those involved in the maintenance and induction of immune responses. This trade-off may explain why stress-induced corticosterone secretion reduces the strength of immune reactions. In many cases, the immunosuppressing effect of corticosterone affects differentially various components of immunity. However, which component is suppressed varies between studies and it remains unclear whether the trade-off in energetic demands accounts for the differential immunosuppressive patterns. In the present study, we investigated whether corticosterone affects differentially two major components of the immune system, namely the constitutive innate and humoral acquired immunity. We used barn owl *Tyto alba* nestlings, implanting half of them with a corticosterone-releasing pellet and the other half with a placebo pellet. To measure the effect of this experiment on humoral immunity we vaccinated half of the corticosterone-nestlings and half of the placebo-nestlings with a solution of four non-pathogenic antigens called 'Tetravac', and the other halves were injected with the control solution PBS. To assess the cost of an elevated corticosterone concentration, we measured nestling body mass growth and resistance to oxidative stress, two key fitness components. Our treatments were successful since implantation of a corticosterone pellet increased blood corticosterone level while the vaccination induced the production of antibodies specifically directed towards Tetravac. Corticosterone significantly reduced the production of antibodies, but it did not affect significantly the constitutive innate immunity. Corticosterone had a negative effect on body mass growth and resistance to oxidative stress demonstrating that it affected negatively key fitness components. Under stressful conditions barn owl nestlings seem to keep the first line of immune defence, the constitutive innate immunity, while an elevated blood corticosterone level negatively affects inducible immune responses. Since we did not find evidence that mounting a humoral immune reaction is costly in terms of growth and resistance to oxidative stress, we suggest that corticosterone may suppress the humoral immune system to a larger extent than the constitutive innate immunity because under stressful situations the risk of immunopathologies is disproportionately higher when mounting an antibody response.

Introduction

As a response to stressful situations, glucocorticoids, such as corticosterone in birds, are secreted to induce behavioural and physiological changes allowing an organism to cope with the demanding situation (Wingfield & Hunt 2002). Previous studies showed that a variety of stressors affect parasite and pathogen defence mechanisms of animals usually leading to a down-regulation of immune responses (El-Lethey, Huber-Eicher & Jungi 2002; Laudenslager et al 1988; Wick *et al.* 1993; but for contrasting results see Dhabhar and McEwen 1996; Irwin *et al.* 1989; Jessop, Gale & Bayer 1987).

A widespread explanation for the stress-induced reduction in immunity is that the induction and maintenance of immune activity is energetically costly and hence trades-off with other demanding functions (Gustafsson *et at*. 1994; Sheldon & Verhulst 1996; Svensson *et al.* 1998; Hanssen *et al.* 2004; Moret & Schmid-Hempel 2000). Thus, the suppression of the immune system during stressful exposures may allow organisms to reallocate resources to other physiological processes, thereby enhancing fitness or immediate survival. In periods of stress, the risk of immunopathology and autoimmune reactions leading to tissue damage may be particularly pronounced and costly (Adams 1996; Råberg *et al.* 1998; Levin and Antia 2001), and thus down-regulation of the immune system can be momentarily beneficial. An additional gain of a reduced immune activity evoked by stress is the avoidance of oxidative stress (von Schantz *et al.* 1999; Svensson *et al.* 1998), because a higher metabolic rate induced by a stressful situation entails the production of free oxygen radicals, which have a damaging effect on cellular processes. Since the immune system is divided into different compartments with a complex network of regulation, stress is likely to differentially affect different components as recently shown in birds (Bourgeon and Raclot 2006; Illmonen *et al.* 2003; Lochmiller, Vestey & Boren 1993).

Here we investigate the effect of moderately elevated plasma concentration of the stress hormone corticosterone on one component of each the innate constitutive and humoral acquired immune systems in barn owl *Tyto alba* nestlings. We chose these two components because the former is the first line of defence while the latter is induced against specific pathogens. We simulated a physiological stress situation by implanting a corticosterone-releasing pellet, which moderately elevates plasma corticosterone level, while other individuals served as a control by implanting a placebo pellet. In this way, the effect of an experimental elevation of circulating corticosterone over several days could be studied without any direct deleterious effect of a natural stressor like food deprivation or disturbance

that induce an elevation of corticosterone (Wingfield & Kitaysky 2002). To assess the effect of corticosterone on humoral acquired immunity, we vaccinated half of the corticosterone-nestlings and half of the placebo-nestlings with a cocktail of four non-pathogenic antigens (Tetravac) and the other halves with a control physiological solution PBS. Our prediction is that corticosterone negatively affects the production of antibodies specifically directed towards the vaccine Tetravac in individuals that were vaccinated and implanted with a corticosterone-releasing pellet. We also tested whether corticosterone suppresses innate constitutive immunity. Natural antibodies in the blood recognise invading particles like foreign blood cells and bind them, thereby initiating the complement enzyme cascade, which results in cell lysis. Thus, agglutination arises from natural antibodies only, whereas lysis reflects the interaction of natural antibodies and complement proteins. Both scores can be interpreted as an index of the strength of the constitutive innate immune system, with higher scores indicating a more effective immune response. Therefore, our prediction is that corticosterone lowers the scores of the haemolysis and agglutination assays in individuals implanted with corticosterone compared with a placebo pellet independently of whether nestlings were vaccinated with Tetravac or not. In addition, we checked the assumption that corticosterone depresses nestling body mass growth and resistance to oxidative stress, two key fitness components.

Methods

Experimental procedure

The study was carried out in Western Switzerland in an area covering 190 km^2 where 150 nest-boxes placed on barns were available for breeding barn owls. We checked boxes regularly for monitoring clutches and hatching dates. In 2006, when the present study was carried out, breeding conditions were the poorest for the last 20 years resulting in only 27 clutches producing only 73 chicks at fledging. For comparison, in 2002 86 clutches gave 285 fledglings. We marked nestlings by clipping off the tip of claws before they were big enough to be ringed with an aluminium ring.

Our experimental design necessitated four barn owl nestlings per nest, and for this reason we selected the four oldest individuals of each nest. Nestlings hatch every 2.5 days resulting in a pronounced within-brood age hierarchy. For this reason, we used only the four oldest nestlings in 21 nests (mean brood size at the first visit was 4.3 ± 0.9, range: 3 - 6). When the oldest chick of each brood was 26.6 ± 2.3 days old (hereafter referred to as day 0),

we selected two individuals per nest to implant a corticosterone-releasing pellet under the skin of the flank. The implant consisted of a biodegradable carrier-binder containing 15 mg corticosterone that releases this hormone during seven days in rats (Innovative Research of America, Sarasota, Florida). The two other siblings served as control by implanting a placebo pellet containing only the biodegradable carrier-binder. The dose of corticosterone in the implants was designed for nestlings with a body mass ranging from 250 to 300 g. Hereafter individuals implanted with corticosterone are denoted 'cort-nestlings' while those implanted with a placebo pellet 'placebo-nestlings'.

Two days after having implanted nestlings, we vaccinated subcutaneously in the neck half of the cort-nestlings and half of the placebo-nestlings with 100 µl of the vaccine Tetravac (TETRAVAC© vaccine, Aventis Pasteur MSD, Switzerland). This vaccine includes a cocktail of antigens (diphtheria 60 UI/mL, tetanus 80 UI/mL, pertussis 50 µg/mL, filamentous haemagglutinin 50 µg/mL, type 1 poliovirus D antigen 80 units/mL, type 2 poliovirus D antigen 16 units/mL and type 3 poliovirus D antigen 64 units/mL) mixed in a solution containing 0.30 mg of aluminium hydroxide which boosts humoral immune responses (Schijns 2000). In the other halves of the nestlings we injected in the neck a phosphate-buffered saline solution (PBS). Hereafter, vaccinated individuals were referred to as 'Tetravac-nestlings' and control nestlings 'PBS-nestlings'. With our experimental design we therefore created four groups of nestlings differing in the intensity of stress and humoral immune stimulation (22 cort- and Tetravac-nestlings, 19 cort- and PBS-nestlings, 18 placebo- and Tetravac-nestlings and 18 placebo- and PBS nestlings). Age at implantation did not differ between the four treatments (one-way ANOVA, $F_{3,73} = 0.98$, $P = 0.41$) neither did rank in the within-brood age hierarchy (one-way ANOVA, $F_{3,73} = 0.58$, $P = 0.63$). Nestlings were weighed at each nest visit on days 0, 2, 4, 12, 17 and 26 post-implantation. Sex of nestlings was identified using molecular markers as explained in Py et al. (2006). Mortality until fledging was neither increased by implantation ($\chi^2 = 0.251$, $P > 0.6$) nor by vaccination ($\chi^2 = 0.717$, $P > 0.4$).

Assessment of plasma corticosterone concentration

We took a 70 µl blood sample by puncturing the brachial vein on day 0 just before implantation, on day 2 just before vaccination, and on day 4 and 12. Samples were collected with heparinised capillary tubes, immediately centrifuged and the plasma stored in liquid nitrogen. After transport to the laboratory, the samples were stored at –20°C until analyses in the next autumn. An increase in circulating corticosterone after an initial stress is detected

after three minutes (Romero & Reed 2005; B. Almasi unpublished data), and for this reason we measured baseline corticosterone level in 160 of the 216 samples (74%) collected within 3 minutes of first opening the nest-box. B. Almasi ran the measurements using an enzyme immunoassay (Munro & Stabenfeldt 1984; Munro & Lasley 1988; Müller *et al.* 2006).

Assessment of humoral acquired immunity

To measure the quantity of antibodies specifically directed against the vaccine Tetravac, we took blood samples on day 2 (just before individuals were injected with Tetravac or PBS) as well as on days 12 and 17. J. Gasparini and K. Stier ran the analyses using a modified enzyme-linked immunoabsorbent assay (ELISA sandwich). As solid phase we used microtitre plates with 8 x 12 wells. Each well was coated with 100 µl of Tetravac diluted 1:50 in PBS and incubated for two hours at room temperature. After washing the plates five times with PBS-tween 0.05%, we added in each well 200 µl of PBS-tween containing 5% of milk. Plates were incubated for two hours at room temperature and then washed five times. Plasma samples were diluted 1:40 in PBS-tween containing 5% of milk and thereof 100 µl were distributed into each well. Plates were incubated overnight at 6°C and washed five times the following day. We saturated each plate with 100 µl of 3'000-fold diluted anti-chicken IgG marked with peroxydase for two hours at room temperature and washed them five times. 100 µl of peroxydase substrate (o-phenylenediamine dihydro-chlorides, 0.4 mg/ml) were distributed and after 15 minutes at room temperature in the dark the reaction was stopped with 50 µl of hydrochloric acid (HCl 1M). Optical density was read at 490 nm with a spectrophotometer, expressing the amount of antibodies directed against the Tetravac vaccine on an arbitrary scale. As a standard, a mixture of numerous positive samples was measured in three dilutions to correct the OD values for a possible plate effect. After calibration with the standard, the repeatability between OD values of the same samples was high within (R^2_{adj} = 0.83, $F_{14,30}$ = 11.04, $P < 0.001$) and between plates (R^2_{adj} = 0.62, $F_{16,34}$ = 4.303, P = 0.002).

Assessment of constitutive innate immunity

We collected blood on day 0 in 65 individuals, on day 6 (70 individuals) and on day 17 (60 individuals). K. Stier determined agglutination and lysis titers with the hemolysis-hemagglutination assay as explained in Matson, Ricklefs & Klasing (2005) with a small modification, i.e. by using 20 µl of plasma, 20 µl of PBS and 10 µl of a 1% rabbit red blood cell suspension. For each sample, we scored lysis and agglutination from the digitised images blind with respect to treatments. The scores of lysis were often 0, indicating that the

concentration of complement proteins in the bird plasma was too low to lyse the rabbit red blood cells under the given concentration of bird plasma. Because of this imbalance, scores of lysis were recorded as either lysis (scores = 0) or no lysis (scores > 0).

Assessment of the resistance to oxidative stress

We investigated whether the corticosterone and Tetravac treatments reduced resistance to a standardized free radical attack. For this purpose, we estimated resistance to oxidative stress by using the KRL® diagnostic test derived from human medicine, adapted to bird physiology (temperature and osmolarity; Alonso-Alvarez et. al 2004). We submitted a whole blood solution to a thermo-controlled oxidative attack where all antioxidants present in blood interact to slow down red blood cell haemolysis. More specifically, 16 µl of the fresh blood were added to 584 µl KRL buffer for the oxidative stress analysis. The resistance to oxidative stress was assessed as the time needed to haemolyse 50% of the red blood cells exposed to a controlled free radical attack ($T_{1/2}$). Thus, higher values of $T_{1/2}$ stand for higher resistance to oxidative stress. R. Piault ran the analysis within two days after taking each blood sample.

Statistics

Data on corticosterone concentration (ln-transformed), resistance to oxidative stress, amount of antibodies specifically directed towards Tetravac and agglutination scores were analysed in R 2.5.1 using (repeated-measures) linear mixed-effect models where nests and individual identities nested within nests were entered as two random factors. As fixed factors we implemented the number of days after the start of the experiment (i.e. when birds were implanted), implantation (corticosterone or placebo) and vaccination (injection of Tetravac or PBS) plus their interactions. We also added the following nestling characteristics: sex, rank in the within-brood age hierarchy, age at the start of the experiment on day 0, and residual body mass extracted from a linear regression of body mass on age on day 0. We also included the interactions of these variables with implantation or vaccination, but because they were not significant, we removed them from the final analyses presented in Table 1. To analyse the effect of implantation on body mass change we did not introduce residual body mass as an independent variable. Lysis scores were analysed with a binary logistic regression in SPSS 14.0. Non-significant terms ($P > 0.01$) were eliminated stepwise backwards. Throughout the paper means are quoted ± S.E.

Results

Baseline corticosterone level

There was a significant effect of corticosterone implantation on baseline corticosterone levels in interaction with day (mixed model, $F_{2,79} = 46.29$, $P < 0.0001$). Before implantation on day 0 baseline corticosterone levels did not differ between the four treatments (two-way ANOVA, $F_{1,69} = 0.291$, $P = 0.591$ overall mean: 8.2 ± 1.3 ng/ml). Two days after implantation (day 2) baseline corticosterone concentrations were about five-fold higher in cort-nestlings than in placebo-nestlings (mean: 45.9 ± 5.1 ng/ml versus 5.2 ± 0.8 ng/ml; two-way ANOVA, $F_{1,42} = 49.776$, $P < 0.001$). Twelve days after implantation baseline corticosterone level did not differ between the four groups (two-way ANOVA, $F_{1,36} = 0.060$, $p = 0.808$) and was back at about the same level as before the implantation in all four groups (two-way ANOVA, $F_{1,112} = 0.060$, $P = 0.806$; overall mean: 7.8 ± 0.7 ng/ml).

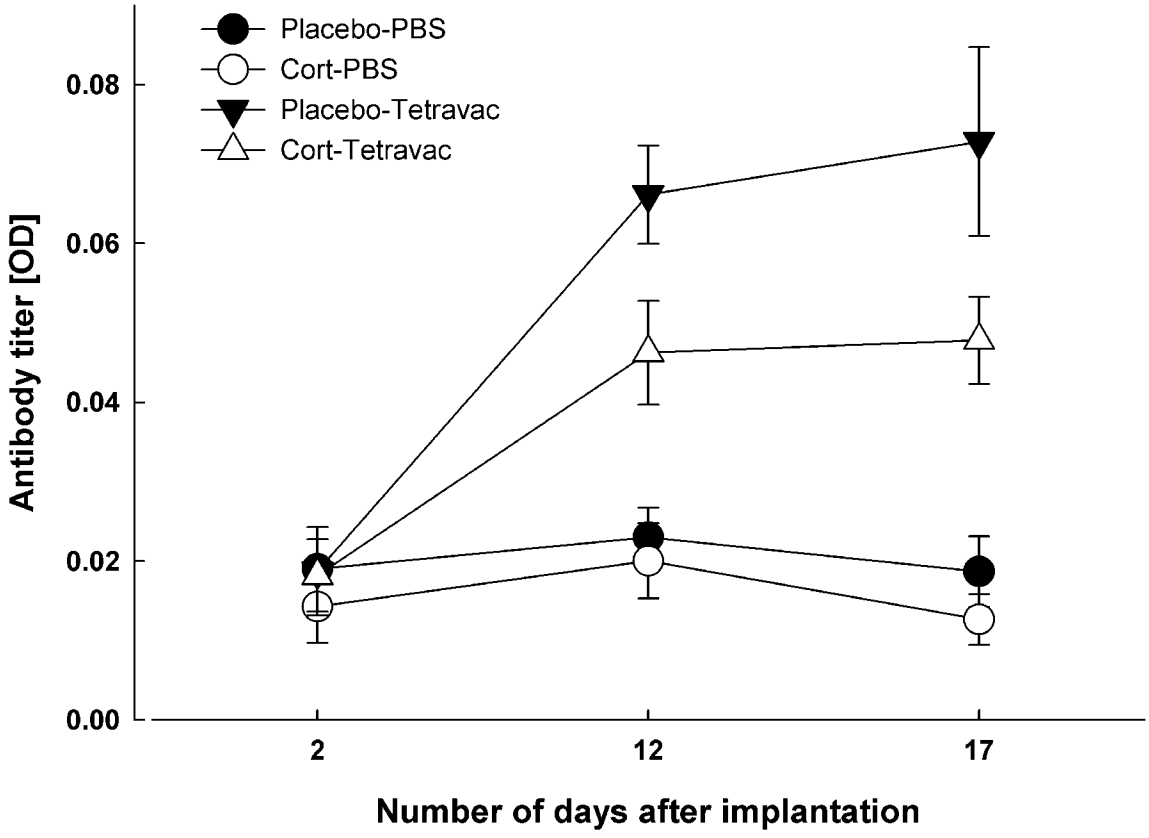

Fig. 1. Effects of corticosterone administration (Cort versus Placebo) and vaccination (Tetravac versus PBS) on the specific antibody production towards Tetravac. Error bars show mean ± 1.0 SE.

Humoral acquired and constitutive innate immunity

Before vaccination on day 2 there was no difference in the amount of antibodies specifically directed against Tetravac between the subsequently injected Tetravac-nestlings and PBS-nestlings, but on days 12 and 17 Tetravac nestlings produced more antibodies directed against the vaccine than PBS-nestlings (Fig. 1, Table 1). Antibody production after vaccination was negatively affected by the implantation of a corticosterone pellet compared to a placebo pellet (Table 1). In Tetravac-nestlings the negative effect of corticosterone administration was pronounced on day 12 and 17, while PBS administration did not result in a difference in antibody titer between cort- and placebo-nestlings (Fig. 1).

The agglutination scores differed between days 0, 4 and 17**,** but independently of the corticosterone treatment and vaccination (Table 1, Fig. 2). The proportion of chicks showing lysis increased from day 0 to day 17 (effect size is 0.566 ± 0.103; binary logistic regression, Wald statistic = 30.1, $P < 0.0001$). Administration of corticosterone and vaccination had no detectable effect on the scores of lysis (Fig. 3).

Body mass growth and resistance to oxidative stress

Before testing the effect of corticosterone on immunity, we verified the assumption that corticosterone had the intended negative effect on physiological processes, here body mass growth and resistance to oxidative stress. Accordingly, during the first two days after implantation nestlings implanted with a corticosterone-releasing pellet lost body mass while placebo-nestlings gained in body mass (Table 1, Fig. 4). From day 2 onwards, body mass change did not differ between cort- and placebo-nestlings. Vaccination had no significant impact on body mass change either alone or in interaction with the corticosterone treatment (Table 1). On day 26, before fledging, body mass did not differ significantly between the four treatment groups (mixed model with implant, vaccination, sex, age on day 0 and the interaction of implantation with vaccination, all $F_{1,42} < 0.80$, $P > 0.35$).

Compared to placebo-nestlings cort-nestlings showed a reduced resistance to oxidative stress on day 12, but not on days 0, 2 and 4 (Table 1, Fig. 5). Vaccination had no significant effect on the resistance to oxidative stress.

Table 5. Mixed model analysis for repeated measures for testing the effect of vaccination and corticosterone implantation on humoral immunity (i.e. production of antibodies specifically directed against the vaccine Tetravac, 210 measurements of 76 nestlings), constitutive innate immunity (i.e. agglutination scores, 186 measurements of 74 nestlings), resistance to oxidative stress (291 measurements of 77 nestlings) and body mass change (354 measurements of 76 nestlings). We present final models after backwards elimination of non-significant terms.

Model	Variable	Estimate (SE)	Df	*F*	*P*
Antibody production	**implant P**	0.007 ± 0.003	1,53	7.79	0.0073
	vaccination	0.002 ± 0.003	1,53	0.39	0.5348
	day		2,130	0.85	0.4313
	day 12	0.006 ± 0.005			
	day 17	0.001 ± 0.006			
	vaccination * day		2,130	19.87	< 0.0001
	vaccination * day 12	0.030 ± 0.006			
	vaccination * day 17	0.040 ± 0.008			
Agglutination scores	**day**		2,110	46.58	< 0.0001
	day 4	1.256 ± 0.131			
	day 17	0.381 ± 0.173			
Body mass change	**implant P**	16.070 ± 3.685	1,53	19.02	0.0001
	day		4,270	18.54	< 0.0001
	day 2-4	20.300 ± 3.199			
	day 4-12	16.190 ± 2.637			
	day 12-17	12.760 ± 2.694			
	day 17-26	10.420 ± 2.587			
	age on day 0	-0.4367 ± 0.084	1,53	27.00	< 0.0001
	implant*day		4,270	5.32	0.0004
	implant P * day 2-4	-16.890 ± 4.700			
	implant P * day 4-12	-17.360 ± 3.858			
	implant P * day 12-17	-17.440 ± 3.941			
	implant P * day 17-26	-16.990 ± 3.784			
Resistance to oxidative stress (T1/2)	**implant P**	-1.409 ± 0.836	1,55	2.84	0.0976
	day		3,208	4.47	0.0046
	day 2	0.685 ± 0.658			
	day 4	0.445 ± 0.7850			
	day 12	-1.585 ± 0.653			
	implant P *day		3,208	7.78	0.0001
	implant P * day 2	-0.275 ± 0.970			
	implant P * day 4	1.232 ± 1.154			
	implant P * day 12	3.909 ± 0.956			

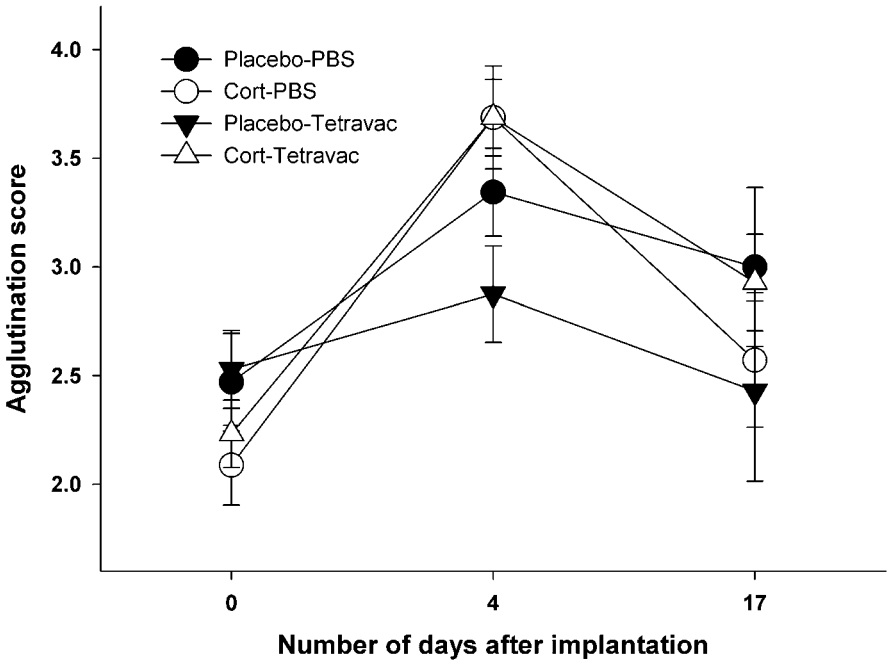

Fig 2. Effects of corticosterone administration (Cort versus Placebo) and vaccination (vaccine versus PBS) on scores of agglutination reflecting the constitutive innate immunity. Error bars show mean ± 1.0 SE.

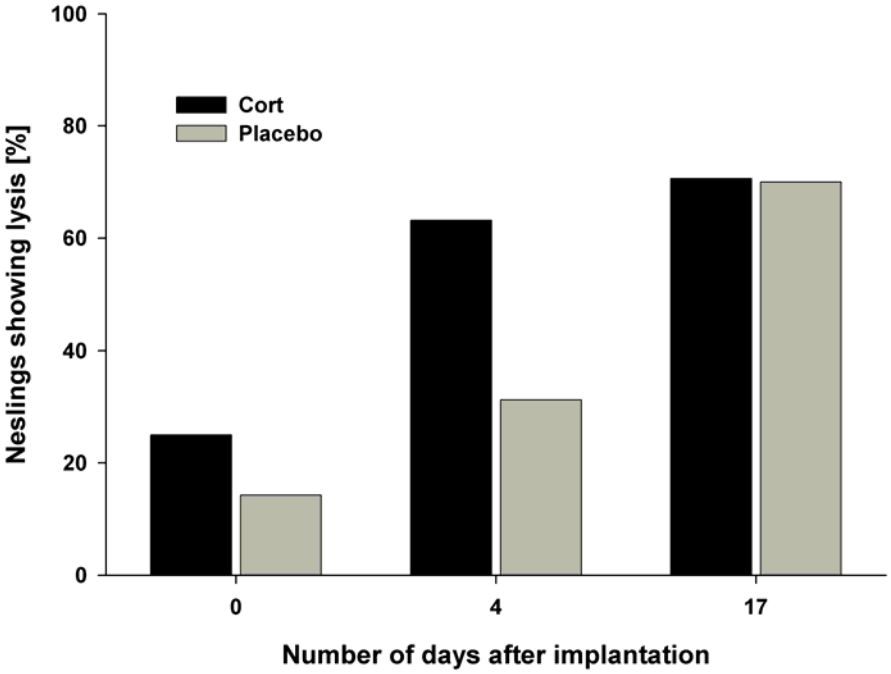

Fig. 3. Effects of corticosterone administration (Cort versus Placebo) on lysis. Bars indicate the percentage of nestlings showing lysis. Higher proportion of lysis indicates a more effective innate immune response.

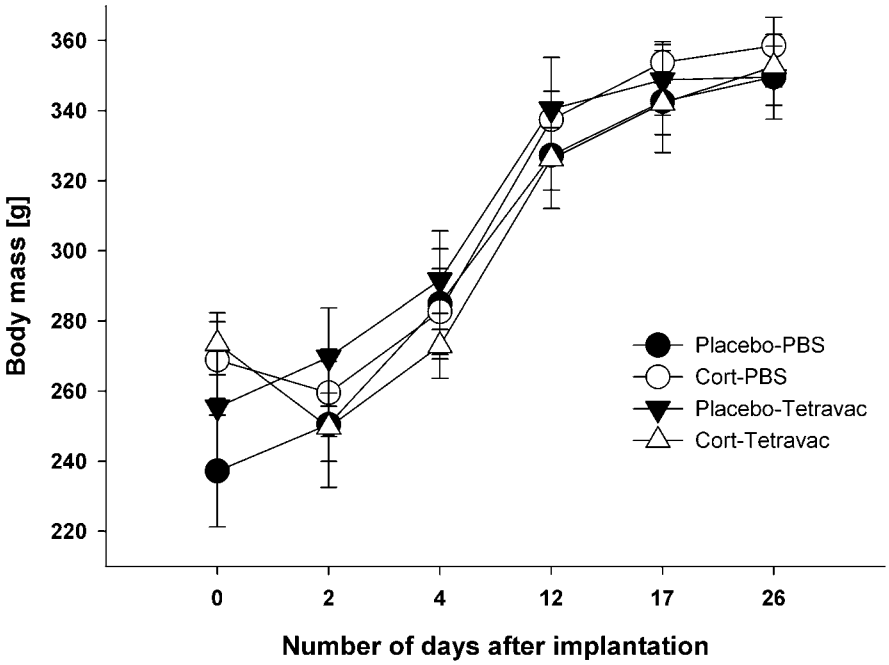

Fig. 4. Effects of corticosterone administration (Cort versus Placebo) and vaccination (Tetravac versus PBS) on body mass growth between days 0 and 26 after corticosterone treatment was applied. Error bars show mean ± 1.0 SE.

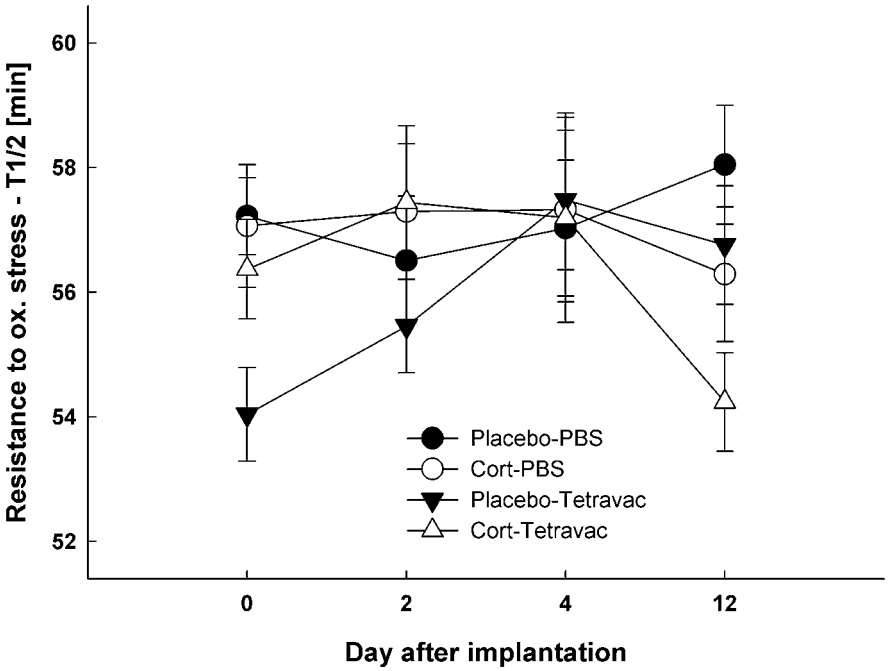

Fig. 5. Effects of corticosterone administration (Cort versus Placebo) and vaccination (Tetravac versus PBS) on the resistance to oxidative stress as measured by T1/2. Error bars show mean ± 1.0 SE.

Discussion

Corticosterone-mediated differential immunosuppression

The present study demonstrated that stress, as induced by administering corticosterone, caused suppression of the humoral acquired immune system, but not of the constitutive innate immune system in barn owl nestlings. The elevation of the corticosterone level to 46 ng/ml represented a moderate increase within the physiological range, as the response to handling stress induced a rise in corticosterone to 60 ng/ml.

The administration of corticosterone reduced the amount of specific antibodies produced against Tetravac in the plasma. Although circulating corticosterone was elevated only during four days, the reduction in the immune response was observed until fledging, indicating that stress causes a long-lasting effect on IgG antibody levels, as also found in rats (Laudenslager et al. 1988), in mice (Moynihan et al. 1990) and in female eiders *Somateria mollissima* (Bourgeon & Raclot 2006). The reduced antibody production against Tetravac through the experimental elevation of circulating corticosterone may be the result of a suppressed antibody production, possibly caused by lymphopenia, i.e. a deficit in B- and T-cells. Indeed, in humans and rats lymphopenia occurs after a session of 2 hours of moderate stress as well as after the injection of corticosterone, an effect that is rapidly reversible (within 24h) after cessation of the stressor (McEwen et al. 1997). However, in the present study corticosterone levels were elevated for several days, and thus such a long period may have profoundly decreased the number of B-cells and in turn the production of antibodies.

Assuming that our two indices of constitutive innate immunity, namely agglutination and lysis scores, were sensitive enough, we did not find evidence for the hypothesis that an elevation in corticosterone level affects this immune component. A differential effect of corticosterone on various aspects of the immune system has already been reported in other bird studies. In female eiders implantation of a corticosterone-releasing pellet significantly decreased the total amount of circulating antibodies but not T-cell mediated immune reaction (wing-web swelling after PHA injection) (Bourgeon & Raclot 2006). Contrasting results were found in northern bobwhite *Colinus virginianus* chicks in which stress due to protein malnutrition reduced T-cell mediated immune reaction measured by wing-web swelling to PHA-injection, but not the humoral part determined by the antibody titers specifically directed to sheep red-blood-cell (SRBC) suspension (Lochmiller, Vestey & Boren 1993). In pied flycatchers *Ficedula hypoleuca,* nestlings of enlarged broods showed reduced T-cell mediated immune responsiveness against PHA, whereas in their male parents corticosterone

levels were elevated but had no effect on the production of specific antibodies against the vaccine SRBC (Illmonen et al. 2003). Thus, birds under stress are able to selectively suppress only certain parts of the immune system and let the other ones unaffected or even enhanced (as demonstrated in mammals by Jessop et al. 1987; Irwin et al. 1989, Dhabhar et al. 1996), possibly as part of a compensatory response (Apanius 1998; Fowles et al. 1993).

Costs and benefits of a differential immune suppression by stress

An immune suppression under stressful conditions entails costs and benefits. Therefore, we would expect that corticosterone affects immune components differentially and which component is negatively affected should depend on the costs and benefits of suppressing each component. An obvious cost of a suppressed immune system is the inability to mount an adequate immune function, especially over such a long period of time as observed in the present study. The risk of infections is increased as well as the progression of diseases already present, leading to serious fitness costs to the point of reduced survival (Apanius 1998; Dalton et al. 1993; Flynn et al. 1993). Barn owl nestlings with elevated corticosterone levels keep the first line of defence, the constitutive innate immune system, which at the same time has hardly any secondary effects. They suppress the humoral acquired immune system, which has a more specific protective function for the body. Tissue injury, a possible risk of an activated immune system, causes the secretion of 'self' antigens and cytokines, which results in further tissue damage and may facilitate autoimmune reactions (Adams 1996; Råberg et al. 1998; Svensson et al. 1998). This potential overshoot of the immune system can be a more important problem to the host than a parasite and, thus, should be prevented or confined by the suppression of immune responses (McEwen et al. 1997). Råberg et al. (1998) proposed that the risk of autoimmune reactions is higher in stressful situations than it is under relaxed conditions.

Immunity is widely assumed to be energetically costly and, therefore, to trade-off with other resource-demanding processes, as caused by a stress response. In this study, we examined the costs of elevated corticosterone levels and the costs of mounting a humoral immune response. Because we elevated corticosterone directly and not via a stressor, we did not confound the direct effect of a stressor (e.g. food restriction in nestlings) with the effect of corticosterone. Corticosterone-implanted nestlings lost, rather than gained body mass during the days of elevated corticosterone and showed a reduced resistance to oxidative stress compared to chicks of the control group. Elevated corticosterone delayed growth temporarily, as in starlings (Love et al. 2005). Because nestling barn owls of the control group reached

maximum body mass at about the age of 44 days (corresponding to day 21 after implantation), the corticosterone-implanted siblings had the opportunity to catch up in body mass and on day 26, shortly before fledging, body mass did not differ significantly between the four groups. Elevated glucocorticoids seem to impair the antioxidant defences (reviewed by von Schantz et al. 1998). Thus, increasing circulating corticosterone as a response to a natural stressor entails clear costs in terms of temporarily reduced growth rate and a reduced resistance to oxidative stress.

In contrast to the effects of corticosterone, we found that a challenge of the humoral immune system (Tetravac injection) did not result in measurable costs in terms of a temporarily reduced growth rate and a reduced resistance to oxidative stress. This result agrees with findings that the energetic costs of an immune response are not high enough to be traded-off against other demanding functions (Amat, Aguilera & Visser 2006; Eraud et al. 2005) and that challenging the immune system did not change the concentration of circulating antioxidants (Hõrak et al. 2006; Cohen, Klasing & Ricklefs 2006). Verhulst, Riedstra & Wiersma (2005) proposed that not the antibody response per se is costly but the maintenance of the system enabling the immune function.

Conclusions

Under elevated corticosterone, barn owls, as other animals, seem to differentially suppress the immune system, i.e. the humoral system but not the innate constitutive immune system. Thereby, birds balance the benefits of a suppression with an acceptable risk of disease susceptibility and progression (Lochmiller & Deerenberg, 2000). The benefits of an immune suppression could be to reduce the energetic costs, as immunity is widely assumed to be energetically costly and therefore to trade-off with other resource-demanding processes. However, as found in our study there was no measurable costs of mounting an immune response under relaxed-conditions (i.e. in placebo-nestlings) and the suppression of the humoral response to an immune challenge under stressful-conditions (i.e. in cort-nestlings) is, therefore, a direct cost of the stress challenge. It seems that avoiding the harmful secondary effects of mounting an immune response under stress may be a major function of the differential suppression of the immune system under stress.

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Synthesis

Synthesis

The present thesis investigates the effects of a short period of chronically elevated corticosterone levels, the main stress-hormone in birds, in an ecological context and draws a link to the mechanisms explaining individual differences in the sensitivity to stress. The barn owl, as model species, provides excellent opportunities to investigate parental investment and nestling development in natural conditions and allows experimental manipulations.

Research on effects of stress has recently received much attention (Sapolsky et al., 2000; Charmandari et al., 2005), but most studies have been done with laboratory animals or animals in captivity (e.g. Kitaysky et al., 2001b; Dong et al., 2007). However, the effects of stress may strongly depend on the context: animals during reproduction do not respond in the same way to stress as non-reproducing animals (e.g. Romero, 2002; Romero et al., 2008) and animals living in a harsh environment react differently to the same stressor as animals living in relaxed conditions (e.g. Romero et al., 2000; Breuner et al., 2003). Therefore, to understand the effects of stress it is very important to study free-living animals under natural conditions and to pay attention to individual differences in the ability to cope with stressful situations. This might help to understand how physiological differences of phenotypes help maintaining different genotypes within a population.

Corticosterone releasing pellets

I chose an experimental approach to manipulate stress hormone levels in free-living barn owls, using the newly available self-degradable pellets releasing corticosterone. These pellets proved to be a powerful tool to elevate corticosterone levels. Pellets are less invasive to use than other implants, because they require only one intervention followed by the release of a given amount of glucocorticoids over a relatively long period of time and do not need to be removed at the end of the experiment. However, the duration of the increase in circulating corticosterone in barn owls and European kestrels was shorter than in mammals. One explanation might be that the higher body temperature of birds resulted in a faster degradation of the pellets. However, the HPA-axis responded strongly to the corticosterone administration resulting in a decreased HPA-axis responsiveness as demonstrated by the decreased endogenous response to an acute stressor of corticosterone-implanted birds. The decreased responsiveness of the HPA-axis lasted longer than the increase in plasma corticosterone levels due to the implants. Furthermore, the increase of circulating corticosterone after implantation of the pellet and the regulation of free corticosterone through CBG varied with environmental conditions and food regimes of the nestlings. These results indicate clearly that the HPA-axis

is not a static system and that its responsiveness depends on the life-cycle stage, but also on the stress-history (e.g. stressful environmental conditions in the recent past) of the animal. Therefore, for an understanding of the biological relevance of effects of stress it is not sufficient to study laboratory animals or animals in captivity. On the contrary, it is crucial to study free-living animals and observe the context the animal is living in; otherwise we most likely miss or misinterpret important aspects of effects of stress.

Effects of stress on parental investment, growth and immune functions

I studied effects of stress in free-living barn owls during two sensitive life-cycle stages, namely reproduction and postnatal development. To understand the consequences of physiological stress for free-living animals and populations it is crucial to apply an increase of glucocorticoid levels as it occurs in nature, e.g. due to inclement weather (Wingfield et al., 1999) or low food abundance (Romero and Wikelski, 2001; Kitaysky et al., 2001a). I elevated corticosterone levels during a short period and looked at the effects during and after this period in terms of parental investment, growth, immunocompetence, and resistance to oxidative stress.

Elevated corticosterone levels in breeding barn owl males resulted in a decreased male investment to the brood, which the female did not compensate for, but were not inhibitory to current reproduction. The decreased provisioning rate of the corticosterone-implanted males resulted in temporarily reduced growth of the nestlings but did not affect survival until fledging. In nestling barn owls a short period of increased corticosterone levels caused a decrease in body mass gain, wing length, and tarsus growth. These reduced growth rates persisted much longer than the time during which corticosterone levels were experimentally elevated; at fledging body mass and wing length of corticosterone-implanted nestlings were still lower than in untreated nest mates. In many bird species fledging body-mass is associated with survival to recruitment (see Magrath, 1991; Schwagmeyer and Mock, 2008). The reduced body mass and wing length at fledging of corticosterone-implanted birds could be disadvantageous at least during the first weeks after fledging. Stressful events during development can have profound, pervasive, and permanent effects on the adult individual, and even on its offspring (reviewed in Metcalfe and Monaghan, 2001). Further studies need to address the question whether a short period of a few days of elevated stress-hormone levels affect recruitment into the breeding population and offspring quality of the next generation.

Elevated corticosterone levels clearly entailed costs in terms of reduced resistance against oxidative stress, which means that more free oxygen radicals are present. Free oxygen

radicals have a damaging effect on cellular processes (Svensson et al., 1998; von Schantz et al., 1999). Elevated corticosterone levels entail a higher risk of infections as well as the progression of diseases because antibody production is reduced (Dalton et al., 1993; Flynn et al., 1993; Apanius, 1998).

All these results together suggest that a short period (only two to three days!) of elevated corticosterone levels influence strongly parental investment and nestling growth and have far longer-lasting effects than the effects that are manifest during the time of elevated corticosterone levels. Such a short period of stress can ultimately shape the phenotype and most likely also influence fitness at adulthood.

Individual differences in the effect of stress and its link to melanin-based coloration

In many instances, environmental heterogeneity is spatial or temporal variation in habitat quality, and conditions prevailing in one habitat or in one year may be more favourable for one genotype, while conditions occurring in another habitat or year may be better suited for another genotype (Kassen, 2002). Thus, one genotype may better perform than another genotype in habitats where parasites are abundant and food scarce, potentially indicating that this genotype better copes with stress (Roulin et al., 2008). A key issue for evolutionary biologists is to understand why one genotype may better resist stress than another. This requires the identification of a candidate gene that can explain variation in reaction norms between genotypes. In this thesis I used the knowledge of the physiological effects of a candidate gene to generate a priori predictions regarding which genotype should be more resistant to stressful environmental factors. I based my predictions on the functional link between melanogenesis and the HPA-axis. The *POMC*-gene codes for different melanocortins, one of which is ACTH whose main action is to induce glucocorticoid release, but also triggers melanin production. Another melanocortin is α-MSH whose main action is to trigger eumelanin synthesis but also plays a role in stress resistance. I used the hypothesis suggested by (Ducrest et al., in review) that eumelanin-based coloration is genetically associated with resistance to stress in vertebrates and formulated the prediction that darker, more eumelanic, individuals are more resistance to stress. This hypothesis is based on a literature review of genetic and pharmacological studies and it is crucial to test experimentally that the degree of eumelanin-based coloration is indeed associated with resistance to stress. In this thesis I performed such experiments in breeding males and nestlings. I found that elevated corticosterone levels affected darker individuals less than whiter ones. More eumelanic males reduced provisioning rates less than whiter males and nestling growth was

less affected if nestlings were darker. These results together support the hypothesis that the degree of eumelanin-based coloration signals the ability to cope with stressful environmental situations. Eumelanin-based coloration, at least in the barn owl, signals resistance to stress and may explain why melanin-based coloration is a mate choice criterion. A further implication of these results is that darker eumelanic individuals may be selected as mates particularly in stressful habitats leading to the interesting possibility that mate choice is context-dependent.

Such a differential effect of elevated corticosterone levels in more or less eumelanic birds can emerge if darker individuals are less affected by elevated corticosterone levels in their behavioural and developmental response or if their HPA-axis is better able to regulate circulating corticosterone levels. Possible mechanisms are that darker individuals reduce exogenous corticosterone through clearance, reduced endogenous production or buffer the increase in free corticosterone through an increase in CBG-capacity, which would lead to lower circulating free corticosterone levels. Nestlings of darker genetic mothers had indeed lower total and free corticosterone levels after corticosterone administration than nestlings of whiter mothers. However, this correlation was only evident in nestlings in 2004 and not 2006. Environmental conditions in 2006 were unfavourable for barn owls, mice and vole populations were very low and not many pairs were breeding that year. Also nestling body condition was better in 2004 than 2006. This suggests that the ability of nestlings of darker mothers to reduce corticosterone levels is condition-dependent and in harsh environmental conditions the differential sensitivity of more or less eumelanic nestlings is not evident anymore. Thus this leads to the suggestion that eumelanin-based coloration can signal resistance to stress but only to some threshold level. Further studies should investigate whether the reduced corticosterone levels of nestlings of darker eumelanic mothers were really due to an increased sensitivity of the negative-feedback mechanism. This can relatively easily be done in the field through injection of dexamethasone, a synthetic glucocorticoid, followed by measuring endogenous corticosterone production. Dexamethasone activates a negative-feedback mechanism (as corticosterone), which regulates endogenous corticosterone production. I, therefore, predict that nestlings of darker eumelanic mothers (and darker eumelanic nestlings) have a lower endogenous corticosterone production after dexamethasone injection as nestlings of less eumelanic mothers (and less eumelanic nestlings). Another experiment should investigate whether the differential behavioural and developmental responses to elevated corticosterone levels also depend on environmental conditions.

We could also demonstrate that elevated corticosterone levels influenced feather phaeomelanin production, which suggests that corticosterone indeed influences the condition-dependent part of melanism and could, therefore, reflect the fitness of the individual during feather production. Unfortunately at the time of corticosterone implantation, the eumelanin-coloured part of the feathers was already developed and I could not investigate the effect of elevated corticosterone levels on eumelanin production. This experiment should be repeated while implanting corticosterone in younger nestlings or better in a more eumelanic species.

The present thesis adds new information on the signaling function of eumelanin-based coloration in the barn owl, as dark owls were significantly better able to cope with an experimental elevation in corticosterone levels than lightly colored owls. Stress sensitivity is an important trait since it influences many other fitness components and when signaled in melanin-based coloration can play a role in sexual selection.

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Curriculum vitae

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Education

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Congress contributions

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Publications in peer-reviewed journals

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